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Iron-Uptake Systems of Vibrio anguillarum

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Presented for the degree of Doctor of Philosophy
in the Faculty of Science, University of Glasgow

Department of Microbiology

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Summary

This thesis is centrally concerned with the characterisation of iron-uptake systems in Vibrio strains isolated from salmonid fish and oysters and the possible role of these systems in pathogenesis. Altogether 23 strains were examined, 13 were Vibrio anguillarum, 3 were V. tubiashi, 1 was V. alginolyticus and 6 were unclassified Vibrio strains. Of the 23 strains, only three contained a plasmid of similar molecular weight (45-50 Mdal) to the virulence plasmid pJMI of V. anguillarum strain 775. Three of the 23 strains could not grow in the presence of transferrin indicating that there was no correlation between plasmid carriage and growth under conditions of iron limitation.

In order to facilitate the serological grouping and further characterise these strains, the LPS of ten V. anguillarum strains were compared by SDS-PAGE and immunoelectroblotting with rabbit antiserum raised against the cell envelope of strain 775. LPS from three strains, strains 1197, 4979 and 775, reacted strongly with this antiserum. All three strains were of serotype J-0-3. Absorbed antiserum against strain 775 which did not react with LPS on immunoelectroblotting still reacted with a further component in the cell envelope of strains 775, 1197 and 4979. This envelope component represents an additional antigen, to the major antigen of LPS, which is specific to serotype J-0-3.

The iron-uptake systems of these ten V. anguillarum strains were then investigated by siderophore analysis, ⁵⁵Fe-uptake and detection plus immunoanalysis of cell envelope proteins associated with iron limitation. Cell envelope proteins produced in the presence of transferrin were detected by an EDTA extraction procedure and PAGE analysis. V. anguillarum strains NCMB6, 636, 827, 4979, 91079 and

2981 produced one additional protein ($M_r = 68-73,000$), strains 775, 1197 and 5679 produced two ($M_r = 73,000$ and $78-79,000$ respectively) and strain 1445 produced three ($M_r = 68, 72$ and $73,000$). Antiserum produced in rabbits to cell envelope fractions of V. anguillarum 775 grown under conditions of iron limitation was absorbed with envelope fractions from cells grown in iron-replete conditions. With this absorbed antiserum, which contained antibodies to the two cell envelope proteins associated with iron limitation, the ten strains were examined on nitrocellulose paper after immunoelectroblotting. Antigenic similarity was seen between proteins associated with iron limitation in all strains. One protein cross-reacted with the absorbed antiserum in strains NCMB6, 636, 827, 1445, 91079, 2981 and 4979 and two proteins cross-reacted in strains 1197 and 5679 indicating that two possible iron-uptake systems existed in V. anguillarum.

The ten strains of V. anguillarum produced three different types of iron-binding compounds when cultured under different conditions. These were (1) a common phenolate-type produced by all ten strains, (2) an hydroxamate-type produced by three strains when grown in Tris-succinate medium (TSM) and (3) a second phenolate-type produced by strains 775, 1197 and 4979, all of which contained a plasmid of 45-50 Mdal molecular weight. The relative affinities of these siderophores, determined by competition for ^{55}Fe was: Second phenolate-type > Hydroxamate-type > Common phenolate-type. However, under these conditions, none removed iron from desferal or purified aerobactin.

Experimental infections of rainbow trout (Salmo gairdneri) were carried out with V. anguillarum strains NCMB6, 775 and 91079, strains which produced different combinations of siderophores in vitro. Only

the common-type siderophore was detected in the kidney and spleen of fish infected with strains NCMB6 and 91079. The hydroxamate-type siderophore produced in vitro by strain NCMB6 was not detected in vivo. However, in the kidney of fish infected with strain 775, both the common and second phenolate-type siderophores were detected, suggesting that the second uptake system was required by strain 775 in vivo and that the iron-uptake system based on the common phenolate-type siderophore was apparently defective.

It was concluded that two independent iron-uptake systems existed in V. anguillarum, one plasmid-mediated, the other chromosomal-mediated and that these systems are produced during infections of rainbow trout.

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Abbreviations

A	Absorbance
BSA	Bovine Serum Albumin
cpm	counts per minute
DNA	Deoxyribonucleic Acid
D	Daltons
EDDA	Ethylenediaminedihydroxyphenylacetate
EDTA	Ethylenediaminetetraacetate
g	gravity
h	hour
kb	kilobase
kD	kiloDalton
K _s	Stability Constant
LPS	Lipopolysaccharide
Mdal	Mega Dalton
M _r	Relative molecular Mass
MRHA	Mannose Resistant Haemagglutination
MSHA	Mannose Sensitive Haemagglutination
mA	milli Ampere
NAS	Nutrient Agar plus Salt
NBS	Nutrient Broth plus Salt
NBST	Nutrient Broth plus Salt and Transferrin
NCP	Nitrocellulose Paper
ONPG	O-nitrophenyl- β -D-galactopyranoside
PAGE	Polyacrylamide gel electrophoresis
Rf	Relative Mobility
rpm	revolutions per minute

rec ⁽⁻⁾	receptor minus strain
rec ⁽⁺⁾	receptor positive strain
SDS	Sodium dodecyl sulphate
sp	species
STET	Sucrose, Triton X-100, EDTA, Tris Buffer
sid ⁽⁻⁾	siderophore minus strain
sid ⁽⁺⁾	siderophore positive strain
TSM	Tris Succinate Medium
TSMT	Tris Succinate Medium plus Transferrin
TCBS	Thiosulphate, citrate, bile salt Agar
TE	Tris, EDTA buffer
V	volts
v	volume
VMM	Vibrio minimal medium
VMMT	Vibrio minimal medium plus transferrin
w	weight

Introduction

1. Historical Aspects

Vibriosis of fish has been known for more than two centuries in Europe and was first described by Banaveri in 1718 (Hofer, 1904) where the etiologic agent was recognised as a pathogen of marine and freshwater fish. Canestrini (1893) first isolated the causative organism of the predominant disease of eels (Anguilla anguilla) in Italy as Bacillus anguillarum. The disease was characterised by haemorrhagic areas in the skin, reddening of the fins and in some cases, the presence of cutaneous ulcers. The bacterium was also pathogenic for sticklebacks, goldfish, frogs and newts, but not for warm-blooded experimental animals. The present name, Vibrio anguillarum, originated from a detailed description of the pathology and bacteriology of vibriosis by Bergman in 1909.

Early interest in the causative agent was stimulated because of its similarity to Vibrio cholerae but the fish pathogen is quite different from V. cholerae (Rucker, 1959). Today, vibriosis has become economically important in the fish and shellfish farming industry and, despite much study, the virulence mechanisms of V. anguillarum and related Vibrio species are not fully understood. From the work of Crosa (Crosa et al., 1977; Crosa et al., 1980; Crosa, 1980; Toranzo et al., 1983; Wolf & Crosa, 1986) an iron sequestering system is thought to be important in the virulence of V. anguillarum and in this thesis the mechanisms of iron uptake have been studied.

2. The Causative Organisms: Vibrio anguillarum and Related Species

Vibrio anguillarum is a halophilic, curved gram-negative rod whose main characteristics are motility and an anaerobic facultative metabolism.

(a) Taxonomy: (i) Vibrio anguillarum

Historically, the marine vibrios which cause vibriosis have been assigned to the taxon Vibrio anguillarum on their biochemical characteristics and deoxyribonucleic acid (DNA) homology (Bergman, 1909, 1912; Schäperclaus, 1928, 1934; Nybelin, 1935; Hodgkiss & Shewan, 1950; Hoshima, 1956, 1957; Bagge & Bagge, 1956; Kishi et al., 1958; Murae et al., 1959; Wolter, 1960; Smith, 1961; Saito et al., 1964; Muroga & Egusa, 1967, 1970; Ross et al., 1968; Cisar & Fryer, 1969; Kiehn & Pacha, 1969; Pacha & Kiehn, 1969). In 1971, Evelyn constructed an archetype of V. anguillarum that would distinguish it from all, or most, of the proposed and recognised vibrios.

However, the above early taxonomic scheme was oversimplified as there is marked heterogeneity among the pathogens causing vibriosis and a second vibrio, not conforming to the archetype of V. anguillarum, has been routinely isolated in Japan and North America.

(ii) Vibrio ordalii

The second, atypical vibrio had been described by several workers (Harrell et al., 1976; Ohnishi & Muroga, 1977; Schiewe et al., 1977; Baumann et al., 1978; Ezura et al., 1980; Tajima et al., 1986) and given a variety of names. These include Vibrio sp 1669 (Harrell et al., 1976), Vibrio sp RT (Ohnishi & Muroga, 1977), V. anguillarum biotype 2 (Schiewe et al., 1977), Beneckea anguillara, biotype 11 (Baumann et al., 1978) and V. anguillarum phenon II (Ezura et al., 1980).

In 1981, Schiewe proposed a new species, Vibrio ordalii for this atypical vibrio, which could be distinguished from the archetypal vibrio on cultural and biochemical characteristics and DNA-DNA hybridisation analysis (Schiewe, 1981; Schiewe et al., 1981). The principal

differences in biochemical characteristics between these two strains or species were as follows.

Biotype 2 strains were unable to:
produce acetylmethylcarbinol (Voges-Proskauer reaction); produce indole; produce arginine decarboxylase; utilise citrate as the sole source of energy and carbon (Simmons' and Christensen's citrate tests respectively); hydrolyse starch; show lipase activity or β -D-galactosidase activity (ONPG test); grow at 37°C and ferment arabinose, cellobiose, glycerol, sorbitol or trehalose.

For these tests the Biotype 1 strains (V. anguillarum) were either all positive or, if variable, the majority of the strains tested were positive.

The above tests are now the main criteria used to distinguish Biotypes I and II and together with DNA-DNA hybridisation analysis showing only 53-69% relatedness, these two causative agents of vibriosis have been divided into two separate species which provide a simpler, more useful taxonomic framework.

In 1984, pathogenic vibrios were subjected to detailed taxonomic analysis by West & Colwell and the phenotypic characteristics which indicated the main differential traits were indole reaction, growth at 37°C and acid production from arabinose, galactose and sorbitol. This overview revealed at least twenty species within the genus Vibrio and attempted to select the important tests for the identification and classification of Vibrio sp for clinical and environmental laboratories.

Other differences in the two species, V. anguillarum and V. ordalii, are also important when considering the preparation of vaccines, pathological studies of laboratory- and naturally-infected fish

and study of the virulence determinants involved in vibriosis.

These will be discussed in more detail later.

(iii) Vibrio tubiashi

Tubiash et al. (1965) showed that certain Vibrio spp were pathogenic for larvae of bivalve molluscs in North America. A number of these strains were placed within the species V. anguillarum and V. alginolyticus (Tubiash et al., 1970) and with the developments in taxonomy and the increasing economic importance of vibriosis in the cultivation of bivalve mollusc larvae, the taxonomy of these were investigated in detail by Hada et al. (1984).

The larvae-pathogenic vibrios were phenotypically distinct from V. anguillarum and other pathogenic vibrios, and this new species was named Vibrio tubiashi. Jeffries (1982) isolated phenotypically similar vibrios in Whitstable, England which were pathogenic to oyster larvae, and Brown (1981) studied two Vibrio strains pathogenic for shellfish larvae which, although biochemically and physiologically similar to V. anguillarum, were morphologically distinct.

(b) Serology of V. anguillarum and V. ordalii

The serology of V. anguillarum is based on thermostable (O) antigens of the lipopolysaccharide (LPS) molecule found on the surface of the outer membrane of gram-negative bacteria (figures 1 and 2). In LPS the antigenic specificity resides in the O-side chain which is composed of many repeating tetra- or pentasaccharide units, usually extending from the cell surface of the bacterium (Nowotny, 1969).

An immunological study of V. anguillarum by Johnsen (1977) showed that LPS from seven V. anguillarum strains was species specific, not being detected in other species examined. Strains of V. anguillarum

Figure 1.

Diagram of a Gram Negative Cell Envelope

From Davis et al. (1980). Reproduced with the
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Publishers Incorporated.

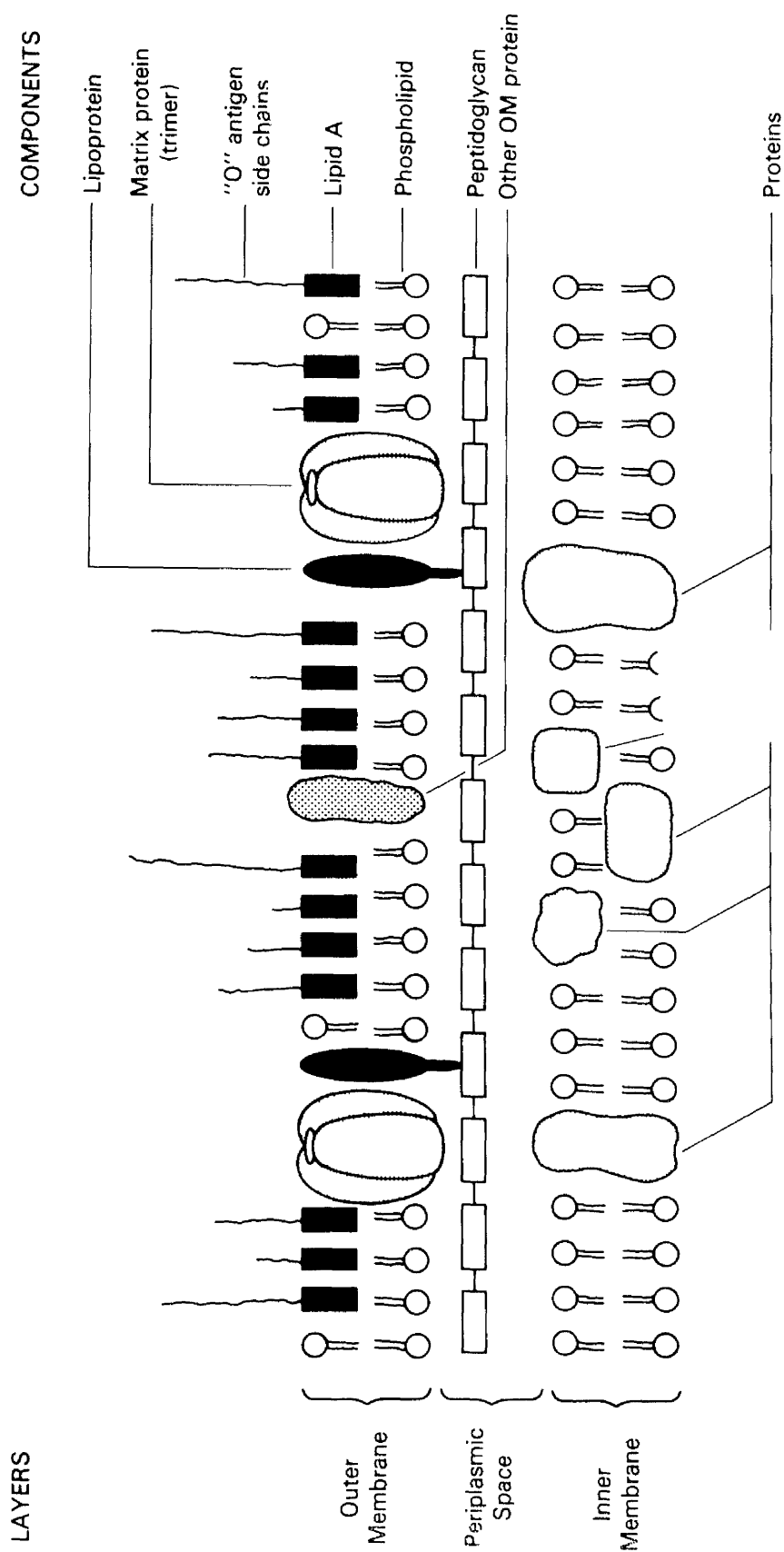
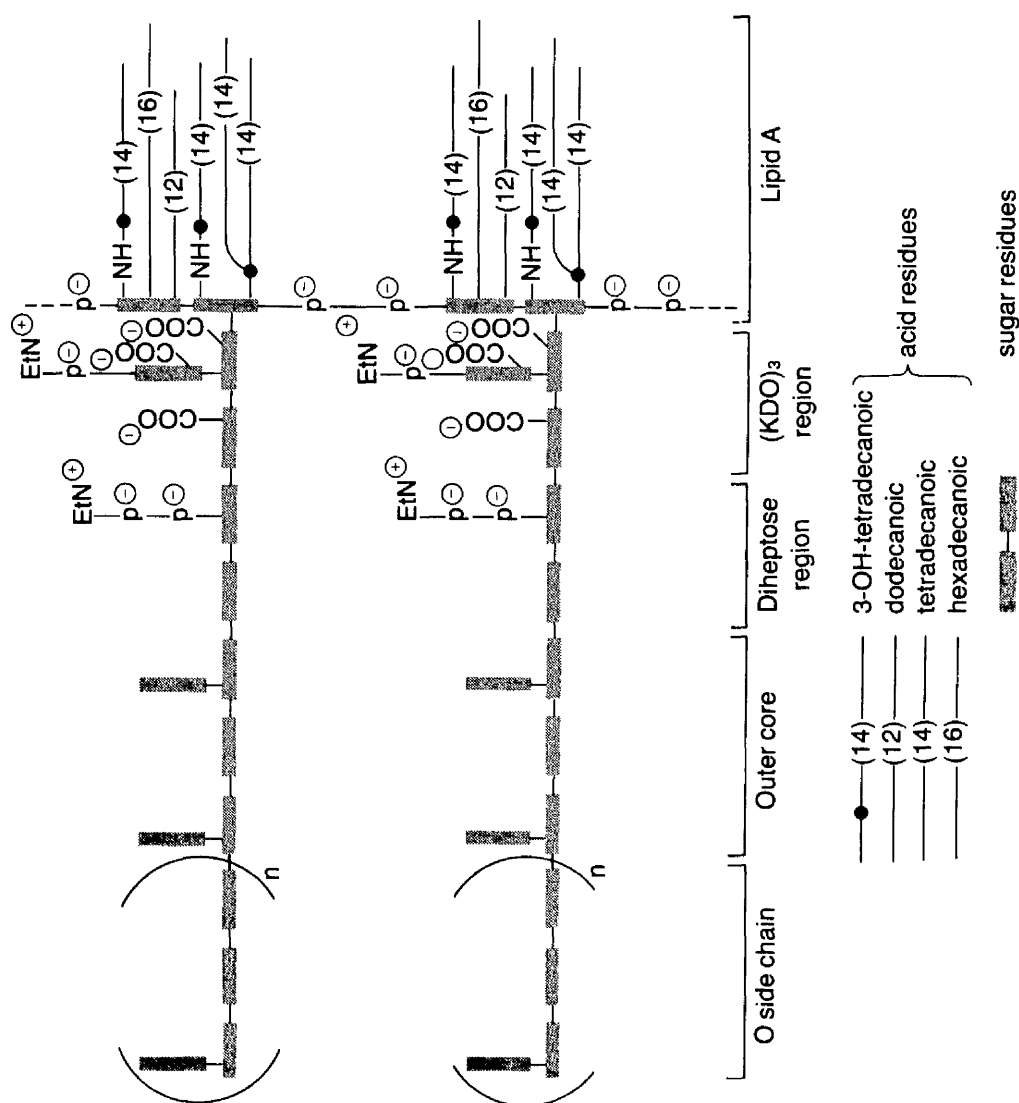


Diagram of a gram-negative cell envelope. Components are listed on the right. The trimers of matrix protein of the Om are associated with lipoprotein and with LPS (of variable polysaccharide length), and lipoprotein is covalently bound to peptidoglycan. Diagram also illustrates some general properties of membranes (see Cytoplasmic Membrane). Phospholipid molecules are illustrated with a **circle** for the polar groups, and a **line** for each fatty acid acyl moiety.

Figure 2.

Schematic Representation of a Molecule of
Lipopolysaccharide.

From Stanier et al. (1977). Reproduced with the
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Schematic representation of a molecule of lipopolysaccharide, containing two cross-linked subunits. The molecular constituents are not drawn to scale. EtN = ethanolamine; KDO = 2-keto-3-deoxyoctonic acid. From H. Nikaido, "Biosynthesis and Assembly of Lipopolysaccharide," in *Bacterial Membranes and Walls*, L. Leive (editor). New York: Marcel Dekker, 1973.

could be placed into three groups according to the serological type of LPS. This suggested that the LPS was the major antigenic determinant of V. anguillarum.

More recently, Chart and Trust (1984) identified surface antigens of V. anguillarum and V. ordalii by an immunoblotting technique. Polyclonal antisera of two common serotypes causing vibriosis in fish in North America demonstrated that antigenic specificity was conferred by LPS, with three serotypes present among the strains tested, confirming the earlier observations of Johnsen (1977). They also observed that the LPS of their type-strains of V. anguillarum and V. ordalii showed some antigenic cross-reactivity. Two distinct LPS morphologies were exhibited within the strains examined (as seen by radiolabelling LPS with ³²P and silver stained polyacrylamide gels) and these corresponded to the different serotypes of V. anguillarum and V. ordalii.

Although these studies demonstrated that LPS was important in the serology of V. anguillarum, Chart and Trust also found that two minor outer membrane proteins of molecular weight 49,000-51,000 were strong antigens. These were common to all strains of V. anguillarum tested and several strains of V. ordalii.

In addition to the above antigens, Sørensen and Larsen (1986) recently suggested that many strains of V. anguillarum possess a polysaccharide capsule, or K-antigen. Both the K-antigen and outer membrane proteins could also be important in serological studies of V. anguillarum or V. ordalii.

A study of the sugar composition of LPS from strains of V. anguillarum isolated in Japan showed that different sugars were present in the O-side chain in different serotypes. Glucose, L-glycero-D-mannoheptose and glucosamine were detected in all LPS's, whereas LPS

of serotypes A, B and C (see table 1) were characterised by the presence of galactose, fructose and rhamnose respectively (Schimizu et al., 1984). Although the additional antigens may be used in future serological studies, LPS is still the major component used to differentiate serotypes from any geographical and environmental source.

(c) Interrelationships Between Serotypes of *V. anguillarum* and *V. ordalii*

Many serological studies on *V. anguillarum* have been reported (Cisar & Fryer, 1969; Pacha & Kiehn, 1969; Conroy & Withnell, 1974; Harrell et al., 1976; Egidius & Anderson, 1977; Johnsen, 1977; Schiewe & Hodgins, 1977; Strout et al., 1978; Gould et al., 1979; Ezura et al., 1980; Johnson, 1980; Kitao et al., 1983; Chart & Trust, 1984; Tajima et al., 1986; Sørensen & Larsen, 1986) but unfortunately, the different terminologies used have rendered the subject confusing (see table 1).

Comparative studies by Ezura et al. (1980) and Sørensen and Larsen (1986) have demonstrated the interrelationships between serotypes found in Japan, North America and Europe. From Table 1 there appear to be four main serotypes: serotypes 2, 4 and 5 occur worldwide, 2 and 4 being the most prominent. Other serotypes have only been found in Japan (serotype 5) or North America (serotype 3). Since predominant serotypes appear in different geographical areas it is important to ensure that the correct serotypes are present in the formulation of vaccines in Europe, North America and Japan.

The study of Sørensen and Larsen (1986) showed seven additional serotypes (04-010), separate from the serotypes isolated worldwide (01-03). Furthermore, their study showed that different serotypes of *V. anguillarum* predominated, when comparing strains isolated from feral or farmed fish and those isolated from the environment.

Table 1. Comparison of Serotyping Schemes for Vibrio anguillarum

Serotype	Sørensen & Ezura et al. (1980)	Strout et al. (1978)	Johnsen (1977)	Harrellet al (1976)	Kitao et al. (1983)	Pacha & Kienn (1969)
1	-	-	-	-	-	-
2	02	J-0-1	569 Group	Group I Type II	A	Group 2 (European <u>Vibrio</u>)
3	-	-	507 Group	Group III	-	Group 3
4	01	J-0-3	775A Group	Group II Type I	C	Group 1 (Northwest American <u>Vibrio</u>)
5	03	J-0-2	-	-	B	-
-	04-010				D-F	

Adapted from Horne (1982)

Serotype 01 was most commonly isolated from cultured salmonids and serotype 02 from feral sea water fish although this serotype could also infect salmonids.. These serotypes have also been described by other workers (see Table 1). Although all ten serotypes were isolated from diseased fish, serotypes 08, 09 and 010 were only reisolated from the environment and this was the first reported serotyping of true, environmental strains of V. anguillarum. The environmental vibrios were of low virulence and serotypes 01 and 02, which were often highly virulent, were not isolated in high numbers from the environment. The other serotypes, 03, 04, 05 and 06 were also commonly found in the environment but had an intermediate virulence between the above groups.

This first recorded interrelationship between serotypes of pathogenic and environmental vibrios may become important in monitoring vibriosis in fish farms and natural fresh or marine water.

Serology of *Vibrio ordalii*.

In the serological studies reported on Table 1, V. ordalii only appeared to contain the serotype 2. This serogroup has been described as 02 (Sørensen & Larsen, 1986), J-0-1 (Ezura et al., 1980), 569 Group (Strout et al., 1978), Group I (Johnsen, 1977), Type II (Harrell et al., 1976), Group A (Kitao et al., 1983) and Group II (Pacha & Kiehn, 1969). The different nomenclature used in these studies again renders the serology of V. ordalii confusing.

3. Vibriosis in Fish and Shellfish

(a) Occurrence and Transmission

In Japan, North America and Europe vibriosis is the most economically damaging disease of cultured marine and fresh water fish (Horne, 1982). A number of Vibrio species have been shown to be

pathogenic for fish, for example, V. alginolyticus, V. anguillarum, V. carchariae, V. cholerae, V. damsella, V. ordalii, V. vulnificus, V. parahaemolyticus and V. piscum (Saito et al., 1964; Farkas & Malik, 1986). According to Rucker et al. (1953) the disease usually appears first in the fish ponds in late April or May and persists through the summer periods when the water temperature increases and stresses the fish population.

How the disease becomes established in a fish farm and the origin of the infective agent are as yet unknown. However, there are two main hypotheses; the breakdown of commensalism or the infectious spread of specific pathogens. Outbreaks of the disease could arise from the random meeting of a susceptible host and a pathogenic strain in the environment or from the failure of the host to suppress a commensal strain which could be potentially pathogenic or in which a mutation has increased virulence (Horne, 1982). The hypotheses require a suppressed or stressed host where the natural immune system is depressed.

Vibriosis is primarily caused by halophilic vibrios and is therefore predominantly a marine phenomenon. *Vibrio* fish pathogens survive poorly in freshwater (Bergman, 1909; Ross et al., 1968) and the freshwater form of the disease therefore should only appear where the causative bacterium has little or no contact with the water during transmission, for example, the feeding of an infected diet (Evelyn, 1971). However, in marine fish culture, transmission could be due to ingestion of contaminated food, adsorption through surface tissues, for example, the gill larvella, or by minor skin abrasions perhaps caused by overcrowding in the fish pond or mishandling the fish. In marine water, the water itself is a continuing potential source of infection.

(b) Pathological and Clinical Symptoms of Vibriosis

There have been numerous reports on the pathological and clinical symptoms of vibrio infections in marine and freshwater fish (Rucker et al., 1953; Saito et al., 1964; Ross et al., 1968; Anderson & Conroy, 1970; Hacking & Budd, 1971; McCarthy et al., 1974; Horne et al., 1977; Harbell et al., 1979; Lewis, 1986) particularly in salmonids where the main symptoms observed were extensive congestion and haemorrhagic lesions throughout the muscular and internal organs, ulceration and bloody areas on the body surfaces, hyperemia of body and fins and bacteremia. A similar pathological pattern was seen in Pacific herring (Clupea pallasii) but in other species of fish the symptoms of the disease vary. Smith (1961) found no single set of disease symptoms common to finnock (immature Salmo trutta) and Horne et al. (1977) recorded an oedematous syndrome in juvenile turbot (Scophthalmus maximus) which had not been described previously; Lewis (1986) noted that in addition to the common characteristics of vibriosis, the intestinal tract of Channel catfish (Ictalurus punctatus) was filled with a clear viscous fluid and, finally, in a study of vibriosis in tropical fish in a freshwater aquarium (Hacking & Budd, 1971) the blister-like lesions, muscle necrosis or exophthalmus described for rainbow trout were not found. In studies of vibriosis in freshwater and marine fish slight differences have been reported in the characteristics of the disease. In a case of vibriosis in rainbow trout (Salmo gairdneri) (McCarthy et al., 1974) held in seawater, no boil-like lesions were seen, a common symptom of vibriosis in rainbow trout held in fresh water (Ross et al., 1968). When peracute vibriosis occurs in very young fish the gross pathological features seen in older fish are

frequently absent (Anderson & Conroy, 1970; Horne et al., 1977).

Mortalities in this case may be due to various forms of toxic shock.

Therefore, since symptoms can vary according to age and species of fish or whether the fish are held in marine or freshwater, great care must be taken in the diagnosis of the disease to differentiate vibriosis from furunculosis, another important fish infection.

Håstein and Smith (1977) have suggested that Vibrio infections can be graded into three categories:

(a) Skin - Ulceration and haemorrhagic skin lesions.

(b) Muscular - Deep haemorrhagic lesions or "boils".

(c) Fins - Haemorrhagic lesions and rotting of fins.

(c) Physiological and Metabolic Changes During Vibriosis

In coho salmon (Oncorhynchus kisutch) infected with a highly virulent strain of V. anguillarum the haematocrit value, haemoglobin content, numbers of red and white blood cells, plasma, total protein, albumin, β -globulin, chloride, sodium, osmolality and alkaline phosphatase levels were all decreased in infected fish compared to controls (Harbell et al., 1979).

The reduction of red blood cells, haemoglobin and haemocrit value were perhaps due to haemodilution, loss through haemorrhage and/or red blood cell lysis. Cardwell and Smith (1971) also found a progressive effect on haemocrit values, haemoglobin, mean cell volume and mean cell haemoglobin in juvenile chinook salmon with vibriosis.

Total plasma protein and albumin concentrations were markedly reduced in infected fish and this was ascribed to loss of urine due to kidney damage, loss of protein through external lesions and reduced protein synthesis due to liver damage (Hunn, 1964).

Diseased fish commonly exhibit an acute stress response with increased blood sugar levels (hyperglycaemia) and this has been found during vibriosis (Harbell et al., 1979; Wedemeyer & McLeary, 1981).

Increases in plasma potassium and several enzymes suggest the occurrence of cell destruction which can bring about muscle damage (Harbell et al., 1979).

The anaemia, leucopenia, cell necrosis and marked increase in serum isoenzymes and potassium levels strongly indicate that substantial destruction of a variety of cell types and tissues occurs by the time the fish are moribund.

(d) Relationships Between *Vibrio* strains and Vibriosis

Host-range specificity has been reported for pathogenic vibrios (Strout et al., 1978; Egidius & Anderson, 1978).

In 1978, Strout et al. reported a variation in virulence of *Vibrio* species isolated from confinement-reared or feral fish when reinjected into coho salmon (*O. kisutch*) reared in freshwater. The presence of highly virulent vibrios isolated from confinement-reared fish compared to the relatively low virulence of *Vibrio* spp originally isolated from feral fish suggested a host-range specificity among vibrios isolated from different environments.

Egidius and Anderson (1978) found that Norwegian reference strains of *V. anguillarum* isolated from salmon and saithe (*Pollachius virens* (L)) differed only in a few of their biochemical reactions, but the strains isolated from salmonids were of high virulence in salmonids but of low virulence in saithe. Similarly, strains isolated from saithe were virulent in saithe but avirulent in salmonids. This again indicates host-range specificity among *V. anguillarum* strains and

Egidius and Anderson (1977, 1978) suggested that perhaps in taxonomic studies V. anguillarum strains could be grouped according to source of origin.

Another factor, important in the taxonomy of Vibrio species, is a difference in the histopathology of V. anguillarum and V. ordalii infections. Ransom et al. (1984) found that bacteremia caused by V. anguillarum occurred in the early stages of infection whereas with V. ordalii, bacteremia developed only in the later stages of the disease.

(e) Vibriosis in Oysters

Oysters, including the species Crassostrea virginica, C. gigas and Ostrea edulis are cultured extensively in North America and other parts of the world.

Microbial pathogens of oyster larvae include fungi and viruses but, more commonly, marine bacteria, especially of the genera Vibrio and Pseudomonas (Brown, 1973; Disalvo et al., 1978; Elston & Leibovitz, 1980). Vibrio species can be highly virulent in shellfish of several species and ages of the host (Brown, 1973; Zebal, 1978; Leibovitz, 1978).

In 1959, Guillard isolated bacteria from moribund larvae and demonstrated that substances in culture fluid inhibited larval swimming. The pathogen was identified as V. anguillarum and subsequent studies also demonstrated vibriosis of cultured larvae (Tubiash et al., 1965, 1970).

V. anguillarum, V. tubiashi and V. alginolyticus have been routinely found in hatchery waters and vibriosis in larvae is considered to develop due to external stresses on the larvae that makes them susceptible to infection. Such factors could include variations in the quality of phytoplanktonic feed algae, variations in oceanic water quality and contamination of hatchery systems with excess organic wastes, which could promote selective growth of pathogenic bacteria.

In the last case, one may speculate that larval disease does not occur unless a critical threshold of the pathogen population is allowed to build up in the hatchery system (Disalvo et al., 1978).

One aspect of vibriosis is the inhibition of larval swimming, causing the "spotting" phenomenon seen in hatcheries, where large numbers of larvae aggregate on the bottom of culture tanks. This would benefit the bacteria, since the bottom detritus in the tanks, consisting of larval faeces and sedimented feed algae, may stimulate bacterial growth and promote infection. In addition, some strains of V. anguillarum can initiate infection from very small inocula (Disalvo et al., 1978; Brown, 1981).

Brown & Losee (1978) described the clinical aspects of induced and naturally occurring epizootics of vibriosis which caused high mortalities, decreased growth and velar abnormalities. Other studies have shown large scale tissue damage, invasion and attachment by bacteria to digestive glands. Mantle tissue was destroyed and velar cells became detached or internally disorganised. In the early stages of the disease food cycling and nutrient utilisation were disrupted. The infection resulted in extensive tissue necrosis and death. Vibriosis can lead to larval mortalities in excess of 90% within 24h of exposure (Elston & Leibovitz, 1980; Nottage & Birkbeck, 1986).

The management of outbreaks of bacterial diseases in oyster hatcheries requires good hygiene and sanitary procedures that minimise the microbial population (Tubiash, 1975), for example, membrane filtration, heat treatment or UV sterilisation of seawater and the use of axenic algal cultures as food. However, microbial infections can continue to occur intermittently. These may be due to temporary lapses in hygiene

(Brown, 1981); alternatively, they may arise because juvenile oysters have been inadvertently stressed during culture in the hatchery (Garland et al., 1983).

4. Control of Vibriosis

The need for an efficient V. anguillarum vaccine became apparent when vibriosis was found to be a limiting factor for intensive fish culture in seawater. It was therefore commercially important to produce a vaccine to prevent mass mortality (Anderson & Conroy, 1970; Fryer et al., 1972).

(a) Development of a V. anguillarum Vaccine

The vaccines for V. anguillarum have been based on bacterins (Fryer et al., 1977, 1978), usually as formalin killed, whole cells, wet-packed whole cells or lyophilized whole cell bacterin. Such vaccines have been used successfully to protect against vibriosis in coho (O. kisutch), chinook (O. tshawytscha), sockeye (O. nerka), chum (O. keta) and pink salmon (O. gorbuscha), rainbow trout (S. gairdneri), Atlantic salmon (S. salar), goldfish (Carassius auratus), angelfish (Pterophyllum scalare) and eels (Anguilla anguilla) (Johnson, 1980; Austin, 1983).

When preparing a V. anguillarum vaccine it is important to take the following into account:

a) The predominant serotype of disease strains in particular areas so that the vaccine is neither wasteful nor ineffective.

b) In many outbreaks of vibriosis, both V. anguillarum and V. ordalii are involved. For maximum protection, a bivalent vaccine including bacterins of both V. anguillarum and V. ordalii has been

advocated (Harrell et al., 1976; Schiewe & Hodgins, 1977; Gould et al., 1979; Schiewe et al., 1981).

The method of delivery of the bacterin is important, the commonest being by injection, oral (inclusion in food), hyperosmotic immersion, direct immersion and spray (shower) (Amend & Johnson, 1981). Immersion and shower vaccination are currently the methods most widely recommended by commercial vaccine companies. For example, the V. anguillarum vaccine Biovax produced by Biomed Research Laboratories, Inc., Seattle, when used in immersion vaccination field tests, showed mortality levels among vaccinates of only 2.2% compared to 27% control mortality over one growing season (Biomed Technical Bulletin No. 2).

(b) Protection Against Vibriosis in Fish

As the protective antigen of V. anguillarum was immunogenic by both immersion and injection methods, even following heating at 100°C, it was suggested that endotoxin was the protective antigen (Harrell et al., 1975; Gould, 1977; Amend & Johnson, 1981).

Coho salmon and rainbow trout are highly resistant to endotoxins from Escherichia coli, Aeromonas salmonicida and V. anguillarum at temperature ranges of 6-18°C (Wedemeyer et al., 1968; Abe, 1972; Paterson & Fryer, 1974). Abe (1972) found that endotoxin from V. anguillarum caused haemorrhages at the site of the intramuscular injection but no mortalities occurred when the endotoxin was injected either intramuscularly or intraperitoneally into juvenile chinook salmon. Wedemeyer et al. (1968) studied several physiological and metabolic changes during challenge with endotoxins of E. coli and A. salmonicida in coho salmon and rainbow trout; they surmised that the metabolic effects of bacterial endotoxins in salmonids were markedly different from those of higher vertebrates.

Paterson and Fryer (1974) showed that coho salmon were resistant to endotoxin of A. salmonicida, which was toxic to mice. Again, Shimizu et al. (1984) showed that the LPS from V. anguillarum had a significant adjuvant and mitogenic effect in mice although salmonids were highly resistant to the endotoxin.

Antibody production and a complement system exist in salmonids (Chiller et al., 1969a,b) but the extent to which they play a major protective role against vibriosis is speculative. Endotoxin from A. salmonicida (1-5,000µg), administered intramuscularly or intraperitoneally, elicited a humoral response in coho salmon (Paterson & Fryer, 1974) and Harrell et al. (1975) showed that in rainbow trout, the protection against V. anguillarum challenge after immunisation was due to serum-specific antibody. Passive immunisation from an immunised fish to a control fish also afforded protection. In vitro, heat stable (perhaps antibody) and heat labile (perhaps complement) components were necessary to prevent growth of V. anguillarum and in vivo, a component of rainbow trout body mucus, indistinguishable from serum immunoglobulin, was detected after immunisation (Harrell et al., 1976) and this may play a significant role in protection. Passive immunisation has also been demonstrated in rainbow trout by transfer of pronephros cells, spleen, peripheral lymphocytes and serum, strongly suggesting an antibody/humoral response in the protection against vibriosis (Viele et al., 1984).

The opsonising ability of fish antibody shown by Griffin in 1983 could bring about lysis or phagocytosis of V. anguillarum. Griffin showed that phagocytosis of Yersinia ruckeri by rainbow trout macrophages occurred more effectively in the presence of specific antibody than in normal serum; more recently, Honda et al. (1986) showed that phago-

cytosis of V. anguillarum by peritoneal macrophages from normal rainbow trout was enhanced by antibody and complement. Treatment of either macrophages or bacteria by antibody also enhanced opsonisation, suggesting that bacteria-antibody complex activated complement in rainbow trout and activated phagocytosis of V. anguillarum.

(c) Good Husbandry and Management

There is a close relationship between husbandry practice and the occurrence of disease in farmed fish. When fish are stressed at certain stages of development, when they are transported and subjected to certain management procedures, or when water quality is poor, then disease frequently results (Roberts & Shepherd, 1974). The location, design and operation of a fish farm can all affect fish health, mainly by influencing "water quality". High ammonium levels, suspended solids, reduced oxygen tensions and high levels of heavy metals can all cause stress which can predispose fish to various secondary microbial infections (Shepherd & Poupard, 1975).

If fish are maintained under conditions of good husbandry and management, for example by:

- (a) Maximum aeration of the water.
- (b) Prevention of stagnation of the water flow.
- (c) Removal of harmful metabolites and fouling.
- (d) Maintaining densities at a safe level.
- (e) Feeding a balanced diet to the fish.
- (f) Regular chemical analysis of the water

then the incidence of microbially-induced disease should be low (Roberts, 1978).

(d) Chemotherapeutic Agents

Fish are a significant dietary component in Japan and, consequently, fish farming has become economically important. Chemotherapeutic agents such as sulfonamides, nitrofurantoin derivatives, tetracyclines, streptomycin and chloramphenicol have been used extensively, and for a short time successfully, to treat infectious fish diseases, especially in culturing ayu (Plecoglossus altivelis).

The emergence of drug-resistant bacteria has caused concern during the past fifteen years. Watanabe et al. (1971) found that considerable numbers of gram-negative bacteria isolated from cultured fish and fish ponds were drug resistant and carried R-factors (extra-chromosomal plasmids encoding drug resistance).

After a large-scale epidemic of vibriosis in 1973 a dominant characteristic of these epidemics was the ineffectiveness of the commonly used antibiotics (Muroga et al., 1974). Aoki et al. (1974) reported that most virulent V. anguillarum strains carried R-factors and this was assumed to be due to selective pressure exerted by the extensive use of chemotherapeutic agents in fish culture.

Other studies in Japan (Aoki et al. 1975, 1980, 1981, 1985) confirmed the high incidence of R-factors in bacteria isolated from cultured fish and fish farm waters. They suggested that the careless and extensive use of chemotherapeutic agents to combat vibriosis and the changes in chemotherapeutics used had increased the prevalence of drug-resistant strains and the change of drug-resistance markers in V. anguillarum. As drug treatment of cultured fish became ineffective in combating vibriosis in Japan, alternative methods of disease prevention were considered.

Strains of V. anguillarum resistant to antibiotics have not yet

been isolated in North America (Mitoma et al., 1984) and great care has been taken not to introduce drug-resistant strains into the environment.

5. Pathogenicity of *V. anguillarum*

(a) Adherence

For many of the pathogenic enteric bacteria the ability to adhere to the surface of the host's cells is an important initial step in pathogenesis (Smith, 1977). Adherence mechanisms of *V. cholerae* have been extensively studied and there are two specific receptors on the mucosal surface of rabbit small intestine, one (fucose-sensitive) on the brush border and the other (fucose-resistant) on the intact mucosae (Freter & Jones, 1976). For *V. anguillarum* the picture is not yet clear. Horne and Baxendale (1983) showed that in slowly developing infections of mature fish, the gut may be an important site of bacterial multiplication and that bacteria adhered to gut sections of vaccinated fish substantially less than to sections from non-vaccinated fish, suggesting that the gut may be an important focal point for multiplication in infections.

The production of pili and flagella have previously been found to have an important role in attachment to mucosal and epithelial surfaces (Swanson et al., 1971; Jephcott et al., 1971; Jones et al., 1976; Swanson, 1977; Jones, 1977, 1980). In 1983, Chart found a correlation between virulence and the possession of one or more flagella, however, the role of these multiflagellate variants in adherence of *V. anguillarum* has not been shown. Production of pili was also noted in two of the *V. anguillarum* strains studied, but their involvement in

virulence could not be determined. Therefore the actual mechanism of adhesion in V. anguillarum is still unclear.

(i) Adherence to Phagocytic Cells

The prompt ingestion and destruction of invading microorganisms by phagocytic cells as they enter the tissues is a fundamental mechanism of immunity by which fish resist disease (Ellis et al., 1976; Rijkers, 1982). Stave et al. (1985) reported the response of striped bass phagocytes to different species of Vibrio for which the virulence for striped bass had been previously determined.

The measurement of chemiluminescence emitted by stimulated phagocytes is a sensitive indicator of oxidative microbiocidal activity of these cells (Klebanoff, 1982; De Chatelet et al., 1982). Generation of bacterial oxygen radicals and chemiluminescence occurs following attachment of bacteria to the phagocytic cell membrane. Stave et al. (1985) showed that virulent V. anguillarum strains elicited a greater response from striped bass phagocytes than did avirulent Vibrio species.

Other investigators, working with E. coli have shown that fimbriate bacteria adhere to mucous membranes and induce a greater chemiluminescence response from human polymorphonuclear leucocytes than the non-fimbriated strains (Bjorksten & Wadstrom, 1982). Bjorksten and Wadstrom suggested that bacteria which adhered well to host tissue also adhered well to phagocytes.

Along with the ability to adhere strongly to fish tissue, Horne & Baxendale (1983) suggested that this ability to elicit a strong response from striped bass phagocytes may be a contributing factor to V. anguillarum virulence.

(ii) Haemagglutination by V. anguillarum

The haemagglutinating ability of bacteria is closely related to the adhesiveness of bacteria to the surface of cells and this is believed to play an important role in the infectivity of pathogenic bacteria. Several workers have studied the haemagglutination patterns of V. anguillarum to erythrocytes and yeast cells (Oishi et al., 1979; Trust et al., 1981; Toranzo et al., 1983; Larsen & Møllergaard, 1984), however their results vary and no distinct pattern was seen. Toranzo et al. (1983) found that pathogenic strains of V. anguillarum isolated on the Atlantic Coast produced haemagglutinins for fish erythrocytes and non-pathogenic strains did not, but this phenomenon was not observed by Trust et al. (1981) who found that both pathogenic and non-pathogenic strains isolated on the Pacific Northwest could agglutinate fish erythrocytes. Larsen and Møllergaard (1984) compared the agglutination patterns of fish-pathogenic V. anguillarum strains and strains isolated from the environment. There was no obvious difference in the distribution of various agglutination types and therefore the role of haemagglutination in the virulence of V. anguillarum has not yet been clearly shown.

In general, the haemagglutinins of V. anguillarum were sensitive to inhibition by D-mannose, suggesting that this carbohydrate or an analogue is part of the receptor on the erythrocytes for adhesion, ie, mannose sensitive haemagglutination (MSHA). Mannose resistance haemagglutination (MRHA) has also been reported for V. ordalii and some strains of V. anguillarum. In E. coli, MSHA is associated with the presence of type 1 fimbriae on the bacteria (Duguid et al., 1979). The structures involved in haemagglutination of V. anguillarum have not yet been studied.

(b) Serum Resistance

In fish, the role of serum in the defence of the host has not been fully defined, but the mechanism for serum killing in salmonids is thermolabile and has a requirement for Mg^{2+} , but not for Ca^{2+} . This suggests that the alternative complement pathway may be involved and have some role in host defence (Chiller et al., 1969; Ellis, 1978; Nonaka et al., 1981; Trust et al., 1981).

The resistance to the bacteriocidal activity of normal salmonid serum may be a virulence determinant of V. anguillarum and V. ordalii (Trust et al., 1981; Toranzo et al., 1983). Loss of serum resistance has been correlated with a marked reduction in virulence in all pathogenic strains tested.

Such resistance has also been shown for A. salmonicida, the causative agent of furunculosis. In this bacterium, the endotoxin and surface A-protein are reportedly involved in serum resistance (Munn et al., 1982).

The existence of a heat-modifiable outer membrane protein has been demonstrated in V. anguillarum (Buckley et al., 1981) and such heat-modifiable proteins are important in the virulence of E. coli, with roles in adhesion, association with leukocytes and resistance to the bactericidal activity of serum (Schnaitman, 1973; Buckley et al., 1981). The possible role of the 33kilo-Dalton, heat-modifiable protein in the serum resistance of V. anguillarum merits further investigation.

(c) Production of Haemolysins

The anaemic response of infected fish with V. anguillarum led Wolke (1975) and Roberts (1978) to suggest that a haemolysin could be involved in the virulence of this bacterium. Many Vibrio strains produce haemolysin (see Table 2) detected by β -type haemolysis

Table 2. Vibrio species Capable of Producing Haemolytic Toxins

Organism and Source	No. of Strains tested
Human Pathogenic <u>Vibrio</u> sp.	
<u>V. vulnificus</u>	58
<u>V. cholerae</u> 01	9
<u>V. cholerae</u> non-01	3
<u>V. fluvialis</u>	3
<u>V. mimicus</u>	1
<u>V. metschnikovii</u>	1
<u>V. hollisae</u>	11
<u>V. alginolyticus</u>	1
<u>V. damsela</u>	2
Fish Pathogenic or Marine <u>Vibrio</u> sp.	
<u>V. ordalii</u>	2
<u>V. aestuarianus</u>	1
<u>V. campbellii</u>	1
<u>V. gazogenes</u>	1
<u>V. harveyi</u>	1
<u>V. natriegens</u>	1
<u>V. nereis</u>	1
<u>V. nigripulchritudine</u>	1
<u>V. pelagius</u>	1
<u>V. proteolyticus</u>	1
<u>V. splendidus</u>	1
psychrotrophic <u>Vibrio</u> sp.	20

From Nishibuchi et al. (1985)

of erythrocytes incorporated into a special blood agar, Wagatsuma agar (Wagatsuma, 1968; Miyamoto et al., 1969). This haemolytic reaction known as the Kanagawa phenomenon is produced by Vibrio parahaemolyticus and V. anguillarum and there is strong evidence that its production is correlated with the pathogenicity of V. parahaemolyticus (Sakazaki et al., 1968; Miyamoto et al., 1969; Chun et al., 1975; Horne et al., 1977; Nishibuchi et al., 1985).

Munn (1978) has shown the production of a thermolabile haemolysin by V. anguillarum in crude supernate which was toxic in eels, but he was unable to demonstrate a correlation between pathogenicity and the production of haemolysin in vivo. In 1983 Toranzo et al. (1983) showed that both pathogenic and non-pathogenic strains produced haemolysins. Therefore, the role of haemolysins in the pathogenicity of V. anguillarum is uncertain.

(d) Production of Exotoxins

A major virulence determinant of V. cholerae is the production of cholera exotoxin. The structure and function of the exotoxin has been extensively studied (Stephen & Pietrowski, 1986). However, no similar exotoxin has been demonstrated in V. anguillarum.

Umbreit and Tripp (1975) demonstrated that V. anguillarum culture supernate was toxic for goldfish and that the potency of the toxic substance was enhanced after heating at 100°C. However, recent investigations have shown extracellular materials of V. anguillarum, which were toxic for both rainbow trout and mice, were inactivated by heating at 100°C or 121°C for 20 minutes. When purified, this material was made up of two fractions, with two protein components in each fraction, carbohydrate being associated with one of these protein components (Kodama et al., 1985).

Other workers have also shown that the production of protease and haemolysin by V. anguillarum (plus V. anguillarum culture filtrate) can be lethal for rainbow trout (Kodama et al., 1984; Moustafa et al., 1984). When purified protease was injected into goldfish it proved lethal (Inamura et al., 1985), suggesting that a protease may be an important virulence factor.

In infections of shellfish by Vibrio species, toxins are also thought to be involved. Disalvo et al. (1978) demonstrated the production of a water-soluble, heat-stable exotoxin by V. anguillarum which inhibited swimming of larval O. edulis. Other studies have also reported the production of heat-labile, extracellular products lethal to oyster larvae (Jeffries, 1983; Brown & Roland, 1984). A toxin of molecular weight 68 kilo-Dalton which was bacteriostatic was purified by Brown and Roland (1984); when the substance was heated it lost toxicity to shellfish but retained bacteriostatic ability.

Nottage and Birkbeck (1986) have identified four toxic-factors from a culture supernate of the shellfish-pathogenic Vibrio NCMB 1338. These included a spat toxin, haemolysin (20 kilo-dalton) and protease (30 kilo-dalton) all of which were thermolabile and a ciliostatic factor (< 5 kilo-dalton) which was heat stable. These factors may have important roles in the virulence of Vibrio species pathogenic to O. edulis and C. gigas larvae.

(e) Ability to Grow Under Iron-Limiting Conditions

This will be discussed in detail in the following sections.

6. Iron and Microorganisms

(a) The Need for Iron-Solubilising Agents

Although iron is relatively abundant in nature as the fourth most commonly found element on earth's surface, the insolubility of ferric iron (Fe^{3+}) at physiological pH makes the acquisition of iron difficult for microorganisms. In an aqueous medium at neutral pH, iron exists as an insoluble polymer, $\text{Fe}(\text{OH})_3$, which has a solubility constant of about 10^{-38} . Therefore, very little is available as soluble Fe^{3+} . At pH 7.0 the solubility of Fe^{3+} is 10^{-17} M which is far too low to sustain the growth of microorganisms (Neilands, 1981; Messenger & Ratledge, 1986). It is apparent that following the advent of O_2 -evolving photosynthesis, most aerobic and facultative anaerobes have evolved a high-affinity pathway for assimilation of ferric iron (Neilands, 1972).

(b) High-Affinity Iron-Uptake Systems

A schematic representation of low and high affinity iron assimilation is shown in figure 3. The high affinity pathway which operates when the availability of free Fe^{3+} is limiting involves a Fe^{3+} -specific carrier, termed a siderophore (Greek: Iron bearer) and cognate receptor, usually found in the outer membrane of gram-negative bacteria (Neilands, 1981, 1984).

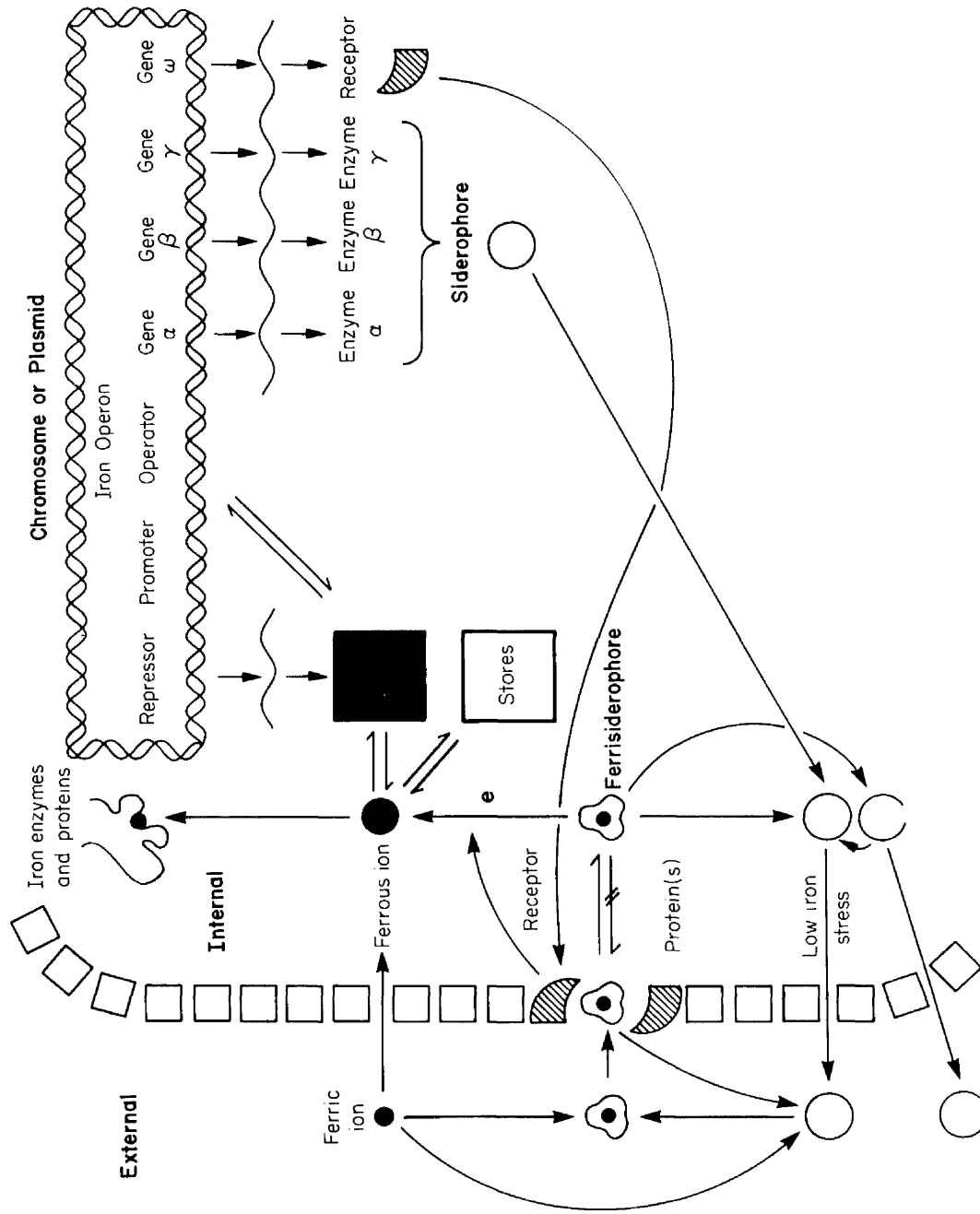
In low-affinity iron assimilation, Fe^{3+} can cross the cell envelope in the absence of siderophore or receptor. Little is known of the low affinity pathway which can be assumed to be widely distributed in microorganisms since depletion of the high-affinity system is not usually lethal (Neilands, 1984). The high-affinity iron assimilation system will be discussed in more detail later in this section.

(c) The Requirement for Iron.

The existence of two oxidation states of iron, ferrous (Fe^{2+})

Figure 3. Schematic Model of Low and High Affinity Iron
Assimilation Pathways

From Neilands (1984). Reproduced with the
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Schematic model of low and high affinity iron assimilation pathways in aerobic and facultative anaerobic microorganisms (slightly modified from a scheme presented earlier).³⁶

and ferric (Fe^{3+}) within the physiological redox range gives iron its ability to act as an electron transporter. Therefore iron is present in cytochromes and non-haem iron electron carriers of the electron transport chain. Ferredoxin, an iron protein, is responsible for the carriage of electrons in the process of nitrogen fixation. Many enzymes contain iron as a co-factor and a list of the possible roles of iron in microorganisms is shown in table 3 (Messenger & Ratledge, 1986).

(d) The Availability of Iron in Host Tissues

In animal tissues most iron is found intracellularly as ferritin, haemosiderin or haem and iron present in serum, lymph, external secretions and milk is attached to high-affinity iron-binding glycoproteins, lactoferrin and transferrin (ovotransferrin also occurs in avian egg white). These proteins have an association constant for iron of about 10^{-36} and they are usually only partly saturated so that little or no iron is available to invading microorganisms. Therefore, to establish an infection under these conditions, potential pathogens must compete for the host's iron by assimilating protein-bound iron or acquiring it from liberated haem (Weinberg, 1978; Bezkorovainy, 1980; Bullen et al., 1978; Bullen, 1981; Griffiths, 1983; Messenger & Ratledge, 1986).

7. Siderophores

(a) Mode of Action of Siderophores in the High-Affinity Iron Assimilation Pathway

Siderophores are low molecular weight (500-1000 dalton), iron-specific ligands whose function is to solubilise iron in the external environment and transport it into the cell. The siderophores are

Table 3. The Role of Iron in Microorganisms

Affected function	Effect
Cell composition	Iron deficiency can cause: growth inhibition, decrease in RNA and DNA synthesis, inhibition of sporulation, changes in cell morphology.
Intermediary metabolism	Processes requiring iron: tricarboxylic acid cycle (aconitase), electron transport, oxidative phosphorylation, nitrogen fixation, aromatic biosynthesis, photosynthesis.
Metabolic products	Biosynthesis of the following products is regulated by iron, porphyrins, toxins, vitamins, antibiotics, hydroxamates, cytochromes, pigments, siderophores, aromatic compounds, DNA and RNA.
Proteins and enzymes requiring iron	Peroxidase, superoxide dismutase, nitrogenase, hydrogenase, glutamate synthase, ribonucleotide diphosphate reductase, aconitase, DAHP synthetase. Cytochromes, ferredoxin, flavoproteins, ferritin or ferritin-like iron storage compounds, iron-sulfur proteins.

From Messenger and Ratledge (1986)

produced by the cells as free ligands and become complexed with iron in the external environment to give the ferri-siderophore complex. They have a very high affinity for Fe^{3+} , with stability complexes in the range of 10^{22} - 10^{52} . The siderophore produced by enteric gram-negative bacteria, enterobactin (enterochelin) has the highest affinity for iron (10^{52}) of known iron-binding compounds (Raymond & Carrano, 1979; Neilands, 1981; Bergeron, 1984).

The ferric-complex is usually transported into the cell via specific membrane receptor proteins in the cell envelope (figure 3). Iron is then released from the ferri-complex by a mechanism which is still unclear, but two hypotheses have been proposed.

(a) Destruction of the siderophore to release the Fe^{3+} molecule.

(b) Reduction of Fe^{3+} by a NAD(P)H-linked siderophore esterase resulting in the formation of Fe^{2+} which has a lower affinity for the siderophore (Langman et al., 1972; Neilands, 1981; Arceneaux, 1983; Messenger & Ratledge, 1986).

(b) The Production of Siderophores by Bacteria and Fungi

A wide range of bacteria and fungi produce siderophores under iron-limiting conditions (see Tables 4 and 5). Indeed, a high-affinity iron-assimilation system has been detected in virtually every aerobic and facultative anaerobic microbial species so far examined (Neilands, 1984).

However, anaerobic bacteria do not require to synthesise siderophores as they utilise ferrous (Fe^{2+}) iron which is freely soluble and available in the environment. The lactobacilli appear to be the only species of bacteria which have no requirement for iron (Archibald, 1983).

Organism	Name and type of siderophore	References
<u>Escherichia coli</u>	enterobactin (P), aerobactin (H)	Brot et al. (1966); O'Brien & Gibson (1970) Stuart et al. (1980)
<u>Aerobacter aerogenes</u>	enterobactin (P), aerobactin (H)	O'Brien et al. (1969); Gibson and Magrath (1989)
<u>Klebsiella pneumoniae</u>	enterobactin (P)	Perry and San Clemente (1979)
<u>K. aerogenes</u>	enterobactin (P)	Williams et al. (1984)
<u>Neisseria gonorrhoeae</u>	gonobactin (H) ?	Yancey and Finkelstein (1981)
<u>N. meningitidis</u>	meningobactin (H) ?	Yancey and Finkelstein (1981)
<u>Pseudomonas aeruginosa</u>	pyochelin (P)	Cox et al. (1981)
<u>Ps. fluorescens-putida</u>	pseudobactin (H)	Teintze et al. (1981)
<u>Salmonella typhimurium</u>	enterobactin (P)	Pollack and Neillands (1970)
<u>Shigella dysenteriae</u>	enterobactin (P)	Payne (1983)
<u>S. flexneri</u>	aerobactin (H)	Payne (1980)
<u>S. sonnei</u>	enterobactin (P), aerobactin (H)	Perry and San Clemente (1979)
<u>S. boydii</u>	Hydroxamate (nn)	Payne (1983)
<u>Vibrio cholerae</u>	Vibriobactin (P)	Payne and Finkelstein (1978)
<u>V. anguillarum</u>	Anguibactin (P)	Grosa (1981); Actis et al. (1986)
<u>V. fluvialis</u>	Phenolate (nn)	Andrus et al. (1983)
<u>V. vulnificus</u>	Phenolate (nn)	Simpson and Oliver (1983)
<u>Azotobacter vinelandii</u>	azotobactin (H), azotochelin (P)	Knosp et al. (1984)
<u>Agrobacterium tumefaciens</u>	agrobactin (H)	Lodge et al. (1982)
<u>Mycobacterium smegmatis</u>	mycobactin (H)	Macham et al. (1977)
<u>M. phlei</u>	mycobactin (H)	Francis et al. (1953)
<u>Corynebacterium diphtheriae</u>	novel siderophore (nn)	Russell et al. (1984)
<u>Bacillus megaterium</u>	schizokinen	Mullis et al. (1971)
<u>Arthrobacter sp.</u>	arthrobactin (H) (terregens factor)	Burton et al. (1954)

H = Hydroxamate; P = Phenolate; nn = not named.

Table 4.

The Production of Siderophores by Bacteria

Table 5. The Production of Siderophores in Fungi and Yeasts

<u>Organism</u>	<u>Name and Type of Siderophore</u>
<u>Ustilago sphaerogena</u>	Ferrichrome (H)
<u>Fusarium roseum</u>	Malonichrome (P)
<u>Streptomyces</u> sp.	albomycin (H)
<u>Actinomyces</u> sp.	Ferrioxamine
<u>(Streptomyces pilosus)</u>	desferrioxamine B (desferal)
<u>Fusarium</u> sp.	Fusarinines
<u>Fusarium dimerum</u>	dimerumic acid
<u>Penicillium</u> sp.	copregen, Fusarinine C
<u>Neurospora</u> sp.	copregen
<u>Rhodotorula</u> sp.	Rhodotorulic acid
<u>Candida albicans</u>	Hydroxamate and Phenolate (nn)
<u>Ascomycetes</u> and <u>Basidiomycetes</u>	Widely produce ferrichromes, copregen

H = Hydroxamate

P = Phenolate

nn = not named

(From Neilands, 1984; Ismail et al., 1985; Messenger and Ratledge, 1986).

The production of siderophores only occurs under iron-limiting conditions which can be induced, in vitro, by several methods, eg., extraction of Fe^{3+} from the medium using 8-hydroxyquinoline in chloroform, adsorption of iron to alumina (Al_2O_3), ion-exchange chromatography, removal of iron by chelation and protein binding of contaminant iron using for example, transferrin. It is also important to avoid iron contamination from glassware which could allow the full growth of microorganisms (Donald et al., 1952; Messenger & Ratledge, 1986).

(c) The Structure of Microbial Siderophores

Microbial iron-chelators fall into two major structural groups; the catechols, or phenolates, and the hydroxamates (Neilands, 1981). Bacteria produce both phenolate and hydroxamate siderophores although no phenolate siderophore has been isolated from eukaryotic cells (Bergeron, 1984).

The catechols consist of cyclic tricatechols, for example, enterobactin, or linear catechols, an example of which is parabactin produced by Paracoccus denitrificans (Tait, 1975). The hydroxamate siderophores comprise a wide-range of structurally different systems including ferrichromes, rhodotorulic acid, mycobactin, fusarine, aerobactin, ferrioxamine and citrate (Gibson & Magrath, 1969).

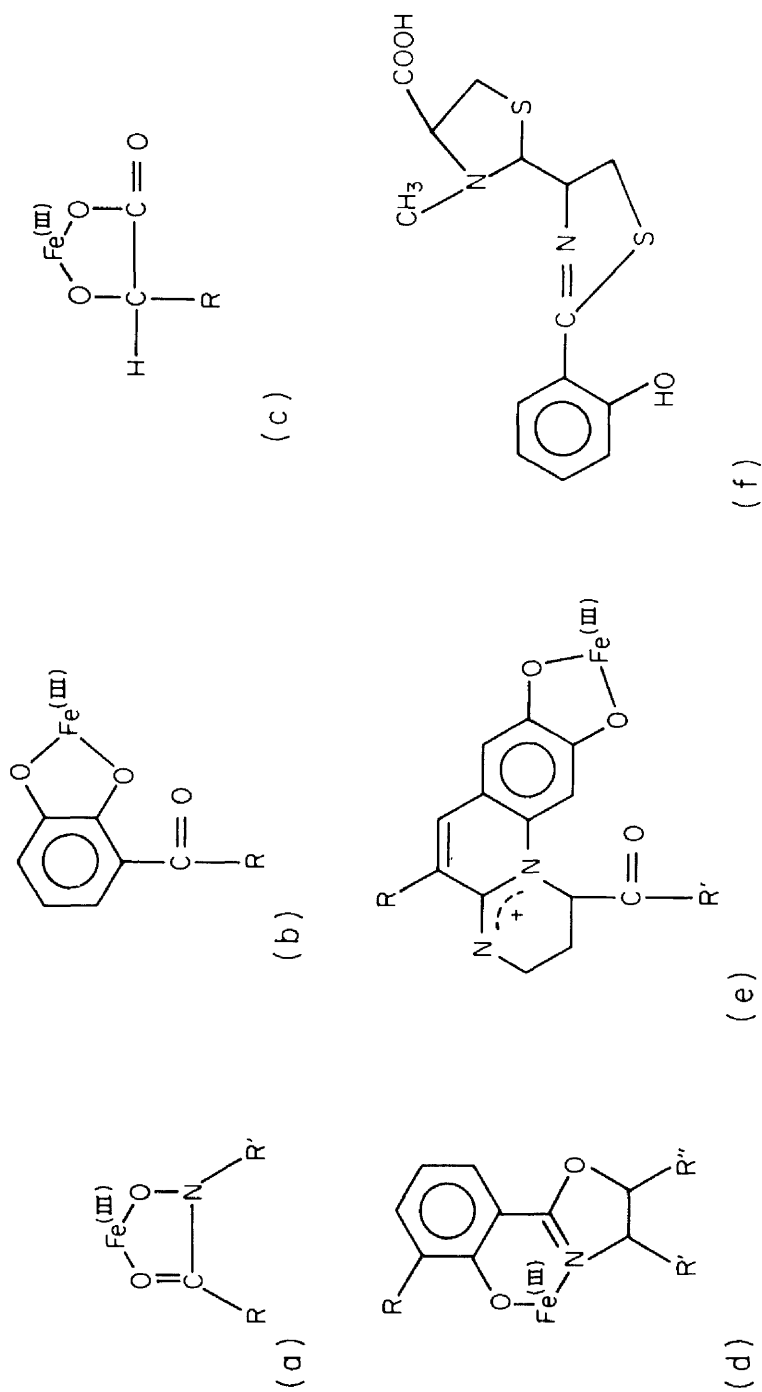
Although these compounds vary widely in their structure, iron is almost always linked to the siderophore via two oxygen atoms to form a bidentate ligand (see figure 4). Thus, these ligands form a high spin hexaco-ordinate (hexabidentate) octahedral complex with iron resulting in extremely stable complexes (Neilands, 1984; Bergeron, 1984).

In addition to complexing with iron, many siderophores form complexes with other metals including aluminium, scandium, gallium, chromium,

Figure 4.

Bidentate Ligand Systems of Siderophores

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Bidentate ligand systems of siderophores (a) Hydroxamate (ferrichromes, rhodotorulic acid, other hydroxamates) (b) Catechol (enterobactin, other catechols) (c) α -hydroxyacid (citrate-containing siderophores, pseudobactin) (d) 2-(2-hydroxyphenyl)-oxazoline (mycobactins, agrobactin, parabactin, vibriobactin) (e) Fluorescent quinolyl chromophore (psuedobactins) (f) 2-(2-o-hydroxyphenyl)-2-thiazolin-4-yl)-3-methylthiazolidine-4-carboxylic acid.

vanadium and copper. Such complexes are not usually as stable as the iron complexes and ferric iron can displace the above metals (Messenger & Ratledge, 1986).

However some metal complexes of enterobactin (gallium and scandium) exhibit antibiotic activity towards pathogenic strains of E. coli and Klebsiella pneumoniae as these complexes can competitively inhibit the formation of the ferri-enterobactin complex (Rogers et al., 1982).

(i) Phenolate Siderophores

Phenolate siderophores can be detected in the culture supernates of bacteria grown under iron-limiting conditions using the colorimetric Arnow Assay (Arnow, 1937) which is selective for aromatic vic-diols with the 3 or 4 positions unsubstituted and not sterically hindered (Barnum, 1977).

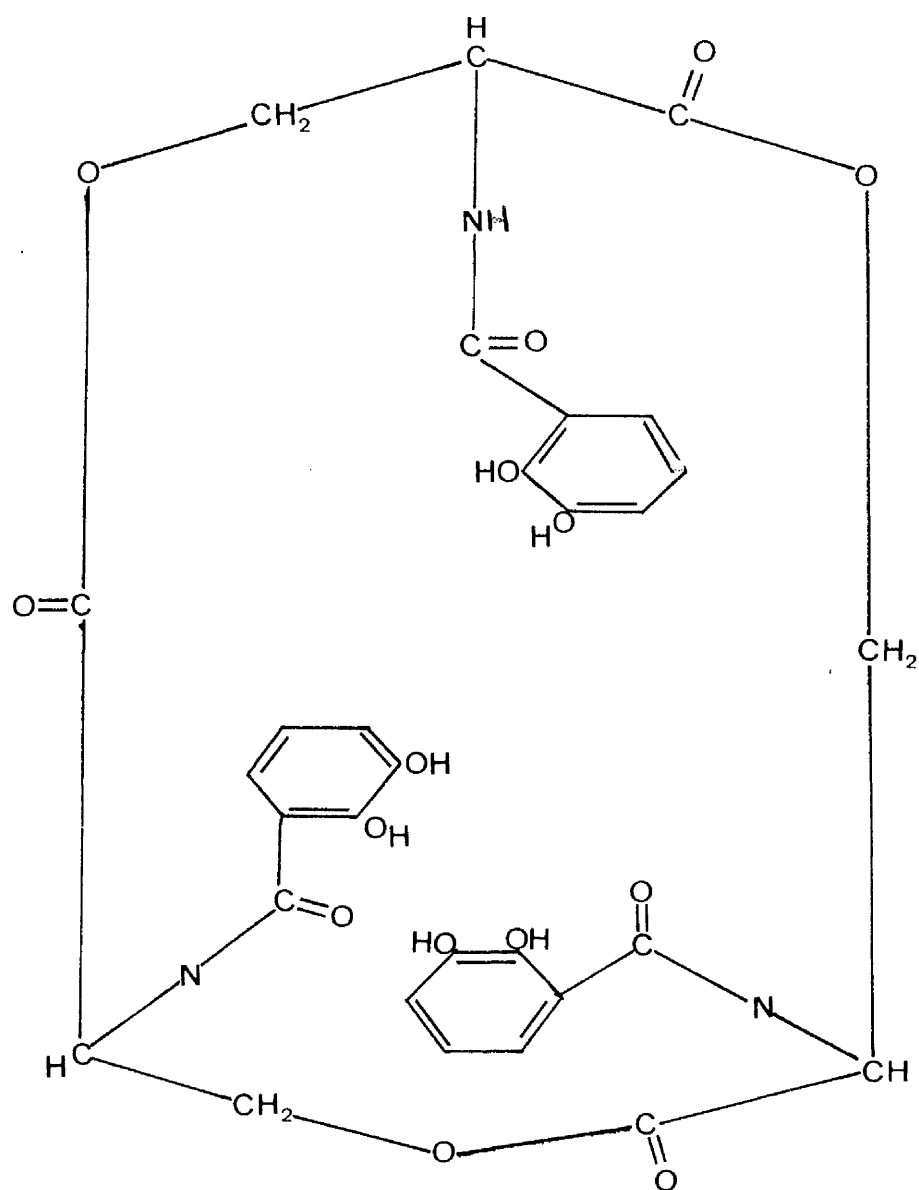
The best known phenolate siderophore is enterobactin, produced by E. coli, Salmonella typhimurium and other enteric bacteria. It is a cyclic triester of 2,3-dihydroxybenzoylserine (see figure 5), and was the first described by Pollock et al. (1970) and O'Brien & Gibson, (1970). Chemical synthesis of enterobactin has since been achieved by several workers (Corey & Bhattacharyya, 1977; Rastetter et al., 1981; Shanzer & Libman, 1983) and is regarded as the prototypical catecholate-type siderophore. Linear catechols include agrobactin produced by Agrobacterium tumefaciens and parabactin from P. denitrificans (Messenger & Ratledge, 1986).

(ii) Hydroxamate Siderophores

Hydroxamate siderophores consist of a group of iron-binding compounds which can differ substantially in structure. They can be detected in culture supernate with the Folin reagent, with acidified

Figure 5.

Structure of Enterobactin from Escherichia coli.



ammonium vanadate or by oxidation with periodate or iodine. The last test, oxidation with iodine is the most popular of the above tests and is known as the Csaky test (Csaky, 1948; Emery & Neilands, 1962; Snow, 1969; Subramanian et al., 1965).

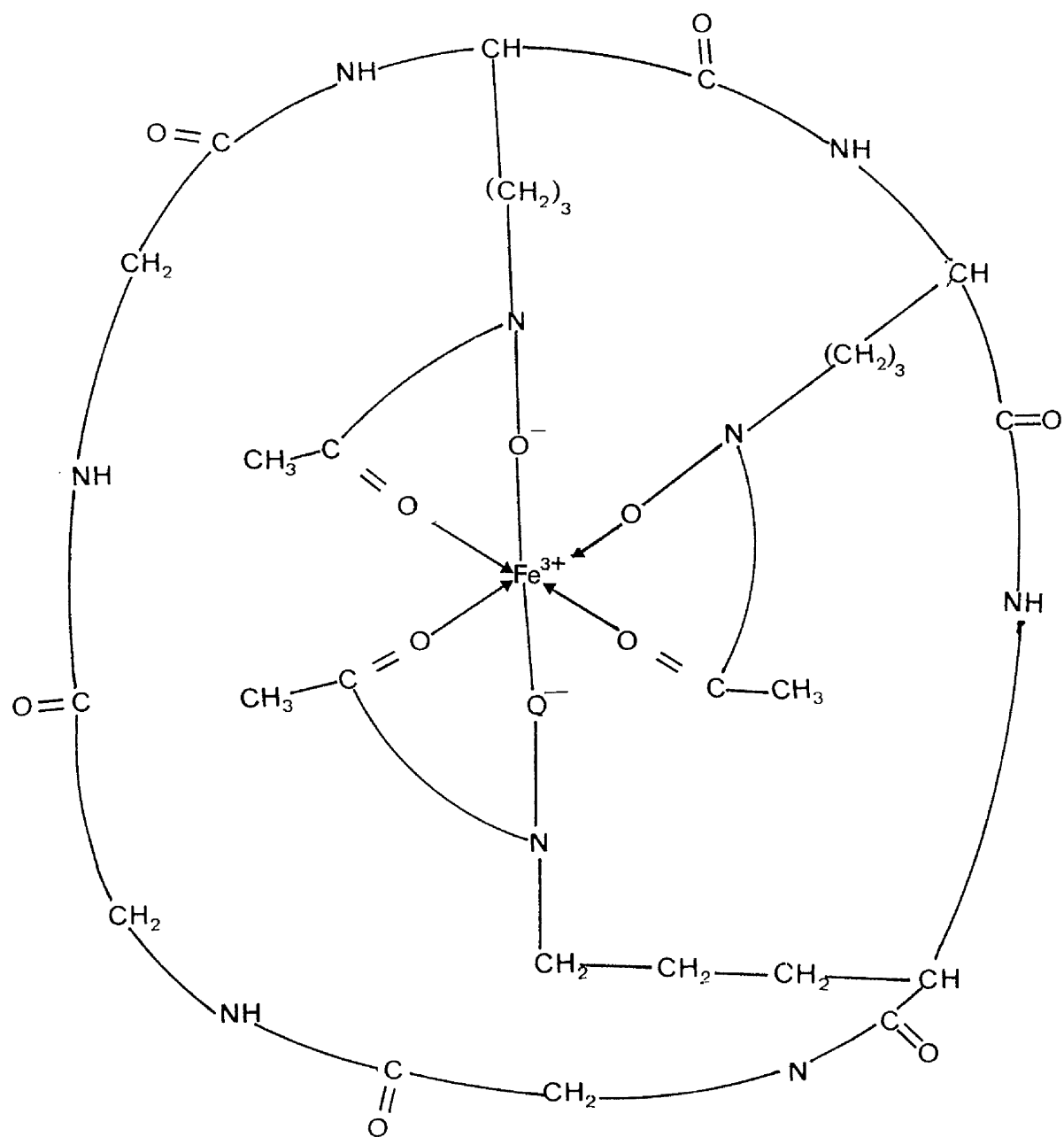
Ferrichrome A, produced by the smut fungus Ustilago sphaerogena, was the first hydroxamate-type siderophore to be reported (Garibaldi & Neilands, 1956) and is now the prototypical hydroxamate-type siderophore (see figure 6).

Ferrichrome-type siderophores are cyclic hexapeptides containing three secondary hydroxamate groups, tripeptides consisting of any of three neutral amino acids; glycine, serine and alanine, and further tripeptides of hydroxyl-amino-ornithine with an acyl substitute on the δ -nitrogen which can be any one of a number of small carboxylic acids (Zalkin et al., 1966; Neilands, 1981). Ferrichrome has been chemically synthesised (Isowa et al., 1974) and the structure of the molecule has been elucidated by X-ray crystallography (Van der Helm et al., 1980).

Variation in amino acid substituents and in the nature of bonding between the hydroxamate group yields a wide range of ferrichrome-type siderophores, for example, malanochrome, albomycin, linear and cyclic ferrioxamines, and fusaranines (see table 5).

Some hydroxamate-type siderophores are known which do not contain three hydroxamate groups. For instance, aerobactin, arthobactin and schizokinen all contain two hydroxamate groups, the third pair of chelating groups being provided by a citrate molecule. Mycobactin also has two hydroxamate groups; here the third pair of chelating atoms are provided by an oxygen atom on the aromatic residue and a nitrogen atom on the oxazoline ring (Messenger & Ratledge, 1986).

Figure 6. Structure of Ferrichrome from Ustilago sphaerogena



Many other groups of siderophores have been shown including ferribactin produced by Pseudomonas fluorescens, rhodotorulic acid and the related derivatives dimerumic acid and coprogen, gonobactin and nocobactin from Neisseira species and the mycobactin-like siderophore from Nocardia species (Yancey & Finkelstein, 1981; Ratledge, 1982; Messenger & Ratledge, 1986).

(d) Possible Uses of Siderophores

Chelation therapy is a widely used treatment for diseases such as iron poisoning, haemochromatosis and related disorders; haemochromatosis involves a progressive increase in iron body stores causing iron deposits in cells of organs, and iron overloading caused by constant blood transfusions. An iron chelator is administered which can form a complex with the iron and then be excreted from the body in urine and faeces. The most effective siderophore used in this treatment is desferrioxamine B from Streptomyces pilosus and this is produced commercially as desferal (Jacobs, 1977, 1980; Callender & Weatherall, 1980; Messenger & Ratledge, 1986).

Siderophores are also important antibiotics. Sideromycins are iron-chelating agents produced by Streptomyces species, for example, albomycin and ferrimycin (Neilands, 1981; Messenger & Ratledge, 1986). A siderophore produced by A. deflectus, desferritriacetylfusarinine C inhibits growth of bacterial species (Anke, 1977) apparently by sequestering iron and making it unavailable to bacteria. Other siderophores such as siderochelin produced by a Nocardia species and a hydroxamate siderophore produced by Pseudomonas alcaligenes have also been shown to have this activity (Barker et al., 1979; Itoh et al., 1979; Liu et al., 1981).

The use of metal analogues of siderophores is another approach used in chemotherapy. Scandium and indium complexes of enterobactin exhibit antibiotic activity towards pathogenic E. coli and K. pneumoniae by competing for binding sites with ferric-enterobactin. (Rogers et al., 1982).

8. Receptors for Ferri-Siderophore Complexes in Enteric Bacteria

The requirement for a receptor is apparent since water-soluble compounds exceeding approximately 500-600 daltons cannot permeate the small, water-filled pores in the outer membrane of gram-negative bacteria (Nikaido, 1979).

The first siderophore receptor to be identified was that for ferrichrome in E. coli (Wayne & Neilands, 1975). This is also the receptor for colicins E and M, phages T₁, T₅ and Ø80 and the antibiotic albomycin (Luckey et al., 1975; Neilands, 1982).

Uptake of Fe³⁺-ferrichrome requires at least two gene products designated Ton A (fhuA) and fhuB. The TonA product is an outer membrane protein (78,000 daltons) and fhuB is a cytoplasmic membrane protein (Pollack & Neilands, 1970; Hantke & Braun, 1975; Braun et al., 1976; Wookey et al., 1981).

FhuB and other gene products, fhuC and fhuD are also involved in the uptake of other hydroxamate siderophores in E. coli, for example, aerobactin, pro-coprogen and albomycin. Extensive genetic studies using albomycin-resistant chromosome mutants with cloned DNA fragments on plasmids have shown the order of the genes on the chromosome to be (fhuA, fhuC, fhuD....fhuB). These mutants involved some defect in the outer membrane protein (fhuA) and also some devoid of peptidases

involved in the release of the antibioticly-active portion of the Fe^{3+} -hydroxamate carrier within the cell (Kadner et al., 1980; Fecker & Braun, 1983; Prody & Neilands, 1984; Köster & Braun, 1986).

A further iron-sequestering system in E. coli is the citrate-dependant iron-transport system which is induced when E. coli cells are grown in a medium containing citrate (Frost & Rosenberg, 1973; Rosenberg & Young, 1974; Hussein et al., 1981). Under these conditions a new outer membrane protein of 80.5 kilo-dalton appears. This protein, designated FecA was considered the membrane receptor for ferric-citrate (Hussein et al., 1981; Wagegg & Braun, 1981). Woodrow et al. (1978) also found another gene product involved in iron-citrate transport, FecB.

Enterobactin synthesis, excretion and uptake have been extensively studied in both S. typhimurium and E. coli (O'Brien & Gibson, 1970; Pollock et al., 1970; Pollock & Neilands, 1970).

The involvement of seven gene products (EntA-EntG) has been demonstrated in enterobactin biosynthesis (Luke & Gibson, 1971; Young et al., 1971; Woodrow et al., 1978; Greenwood & Luke, 1978, 1980, 1981). Three enzymes, the products of genes entA, entB and entC, catalyse the conversion of chorismic acid to 2,3-dihydroxybenzoic acid and a further four enzymes (entD-G) are involved in the condensation of 2,3-dihydroxybenzoic acid and L-serine to enterobactin.

Two additional genes (fes and fep) have been mapped. The fes gene codes for an esterase which degrades enterobactin to 2,3-dihydroxybenzoyl-serine to release the chelated iron (O'Brien & Gibson, 1970; Langman et al., 1972). The fep gene encodes the outer membrane receptor for enterobactin (Cox et al., 1970; Hollifield & Neilands, 1978). Studies with sphaeroplasts have shown that the fep mutation can be of two classes, fepA and fepB (Ichihara & Mizushima, 1978; Wookey & Rosenberg, 1978).

FepA mutant sphaeroplasts still transported ferric enterobactin whereas fepB mutant sphaeroplasts did not, suggesting that the fepA product was an outer membrane protein and fepB a cytoplasmic membrane component. The ferric-enterobactin receptor was of molecular weight 81 kilo daltons.

Further genetic studies by Laird and Young (1980) and Pierce *et al.* (1983) complemented the feuA, feuB and ent genes and by transposon mutagenesis showed the genes to be distributed across a 26 kilobase region of DNA.

The last iron-sequestering system to be described in E. coli was found to be plasmid-coded (Williams, 1979; Williams & Warner, 1980). This plasmid, ColV, which also codes for the production of colicin V, enhanced the virulence of E. coli strains responsible for generalised infections and deaths in livestock and humans (Braude & Siemieski, 1965; Smith & Halls, 1967; Smith, 1974; Smith & Huggins, 1976, 1977; Neilands, 1982; Crosa, 1984). ColV plasmid was shown to code for a hydroxamate-type siderophore, aerobactin, and an outer membrane-bound receptor (iutA) of 74 kilo dalton molecular weight (Stuart *et al.*, 1980; Warner *et al.*, 1981; Van Tiel-Menkveld *et al.*, 1981; Braun, 1981; Stuart *et al.*, 1982; Grewel *et al.*, 1982; Bindereif *et al.*, 1982). This is also the receptor for the bacteriocin cloacin produced by Enterobacter cloacae DF13 (Oudega *et al.*, 1979; Van Tiel-Menkveld, 1981; Bindereif *et al.*, 1982; Van Tiel-Menkveld *et al.*, 1982).

The expression of aerobactin uptake in E. coli has been studied using recombinant derivatives containing the iron-uptake regions in vector plasmids (Krone *et al.*, 1983a,b; Bindereif & Neilands, 1983, 1985). This indicated that uptake of ferric-aerobactin required the production of aerobactin and two polypeptides, a 74 kilo dalton membrane

bound receptor protein and a 50 kilo dalton protein involved in the binding and transport of ferric-aerobactin at the cytoplasmic membrane. This aerobactin-gene complex was about 8 kilobases in length.

In addition to the genes coded on the colV plasmid four chromosomal genes - fhuA, fhuB, tonB and exbB involved in aerobactin-uptake have been described (Braun et al., 1982).

Hantke and Zimmerman (1981) and Neilands (1982) reported that two gene products tonB and exbB were required for the uptake of iron by some or all of the high-affinity iron-transport systems. The tonB gene product was reported to be a 36 kilo dalton molecular weight protein thought to be required for transport across the inner membrane and/or physical association of outer and inner membranes. It is required by all iron-sequestering systems and is probably located in the cytoplasmic membrane (Wookey & Rosenberg, 1978; Postle & Reznikoff, 1979; Plastow & Holland, 1979; Weaver & Konisky, 1980).

The exbB gene product is required for the uptake of both ferrichrome and vitamin B₁₂ (Hantke & Zimmerman, 1981).

9. Regulation of Iron-Sequestering Systems in Enteric Bacteria

The production of both siderophores and their cognate receptors is regulated by the availability of iron (McIntosh & Earhart, 1977) and mutants defective in iron regulation (the mutation is designated fur = ferric iron uptake regulation) have been isolated in S. typhimurium and E. coli (Ernst et al., 1978; Hantke, 1981). These mutants overproduced the outer membrane proteins and were constitutive for all iron-uptake systems tested: Ferrichrome, ferric-citrate, ferric-enterobactin and aerobactin (Hantke, 1981; Braun & Burkhardt, 1982).

By genetic analysis using, restriction analysis, cloning into plasmid pACYC 184, Tn100 mutagenesis and complementation studies Hantke (1982, 1984) attempted to map the fur gene and reveal the type of iron regulation exerted by this gene. In minicells an 18 kilo dalton protein was identified as the fur gene product and the fur gene was mapped on the E. coli chromosome at approximately 15.5 minutes. However, recent work has suggested that another locus could be mutagenised to yield a fur⁽⁻⁾ phenotype, therefore more than one locus may exist in the regulation of iron-uptake (Bagg & Neilands, 1985).

Hantke (1982) has suggested that the regulation of iron-uptake by the fur gene product is under negative control, perhaps due to direct interaction between iron ions and the fur gene product protein.

For a summary of the proteins involved in iron uptake in E. coli see Table 6.

10. Importance of Iron-Sequestering Systems in Microbial Virulence

A common and essential factor in all infections is the ability of the pathogen to multiply in the host and this is greatly influenced by the availability of iron (Griffiths, 1983). Susceptibility to experimental infection can be enhanced by the addition of iron and iron compounds with injections of a variety of different bacterial pathogens, for example: Aeromonas hydrophila (Miles et al., 1979), Clostridium oedematis (Miles et al., 1979), Corynebacterium renale (Henderson et al., 1978), E. coli (Bullen et al., 1968a), K. pneumoniae (Miles et al., 1979), Listeria monocytogenes (Sword, 1966), Neisseria gonorrhoeae (Payne & Finkelstein, 1975), Neisseria meningitidis (Calver et al., 1976), Pasteurella septica (Bullen et al., 1968b), Pseudomonas aeruginosa (Forsberg

Table 6. Some Genetic Functions Required by Iron Assimilation Systems
in Escherichia coli K12

Gene	Map locus (min)	Product/Function
<u>tonA</u> (<u>fhuA</u>)	3	Ferrichrome receptor
<u>fhuB</u> , C, D	3	Hydroxamate siderophore utilization
<u>fhuE</u>	16	Ferric coprogen and ferric rhodotorulate utilization
<u>fiu</u>	18	Induced at low iron, function unknown, not in <u>E. coli</u> B
<u>entA</u> , B, C, D, E, F, G, <u>fes</u> , <u>fepA</u> , B	13	Biosynthesis and transport of enterobactin
<u>fecA</u> , B	7	Ferric citrate utilization
<u>tonB</u>	27	Transport of siderophores and B12
<u>cir</u>	43	Colicin Ia receptor, induced at low iron
<u>exbB</u>	64	Transport of siderophores
<u>fur</u>	?	Constitutive siderophore synthesis
<u>iuc</u> , <u>iut</u>	pColV	Biosynthesis and transport of ferric aerobactin

From Neilands (1984)

& Bullen, 1972), S. typhimurium (Kaye et al., 1965), Staphylococcus aureus (Gladstone & Dalton, 1971), V. cholerae (Ford & Hayhoe, 1976), V. vulnificus (Wright et al., 1981), V. anguillarum (Crosa, 1980) and Yersinia pestis (Jackson & Burrows, 1956).

Under normal conditions iron is not freely available in the body fluid of the host, therefore bacterial pathogens must possess mechanisms for assimilating protein-bound iron or for acquiring it from liberated haem in vivo. The involvement of iron-sequestering systems (including siderophore production and outer membrane receptors) in the virulence of selected pathogenic bacteria will now be discussed in more detail.

(a) Pathogenic Enteric Bacteria

The significance of high-affinity iron-sequestering systems in infections of the enteric bacteria E. coli and S. typhimurium have been extensively studied.

(i) Aerobactin- and Enterobactin-Mediated Iron Assimilation in Escherichia coli Infections

The plasmid-mediated aerobactin iron-transport mechanism was demonstrated in invasive strains of E. coli, when the ability of these bacteria to grow under iron-limiting conditions was correlated with the enhanced ability to cause septicaemic infections in the animal host (Williams, 1979).

In recent years clinical isolates of E. coli have been examined for the presence of aerobactin. The incidence of aerobactin-positive strains of E. coli from the blood was greater than the incidence of these strains isolated in other sites within the host (Montgomerie et al., 1984). Patients with septicaemia, pyelonephritis, and symptomatic and asymptomatic urinary tract infections also showed a higher incidence of

aerobactin-positive strains than among normal human faecal isolates (Carbonetti et al., 1986). These results suggest that the presence of aerobactin is a significant factor in the invasion of the bloodstream and pathogenesis of urinary tract infections by E. coli. Aerobactin iron-transport genes have been shown to occur commonly in the chromosome of different clonal groups of human invasive strains of E. coli K1 and other E. coli isolates (Valvano & Crosa, 1984; Bindereif & Neilands, 1985; Valvano et al., 1986).

It has also been shown that aerobactin has physiological and regulatory features which result in a more effective provision of ferric ions for bacterial growth than does enterobactin, even though aerobactin has an association constant lower than that of ferric-enterobactin or transferrin, and this may give a selective advantage to aerobactin-positive, invasive, strains of E. coli in host tissue (Neilands, 1981, 1983; Williams & Carbonetti, 1986).

When grown in vivo, pathogenic E. coli can express four new outer membrane proteins of molecular weight 83, 81, 78 and 74 kilodaltons; these proteins also appear when E. coli strains are grown in iron-deficient media containing ovotransferrin, suggesting the importance of the iron-binding receptors in vivo (Griffiths et al., 1983). The presence of antibodies reacting against the iron-regulated outer membrane proteins in E. coli, including the ferric-enterobactin receptor protein (81 kilodalton) can be found in sera obtained from normal rabbits, mice, guinea pigs and humans. In the majority of laboratory and clinical isolates of E. coli, the molecular weight and antigenic properties of the ferric-enterobactin receptor protein were highly conserved (Griffiths et al., 1985; Chart & Griffiths, 1985). Thus, it

follows that the antibodies in normal sera will react with the ferric-enterobactin receptor of most pathogenic E. coli strains and the efficiency of the antibody may question the importance of the enterobactin-mediated iron-sequestering system in vivo.

Although enterobactin has been isolated from the peritoneal washings of guinea pigs lethally infected with E. coli, suggesting an important role in the virulence of E. coli infections, naturally occurring antibodies to enterobactin in normal human serum can inhibit ferric-enterobactin uptake and therefore may limit the effectiveness of enterobactin-mediated iron-assimilation in vivo (Griffiths & Humphreys, 1980; Moore & Earhart, 1981). These observations may explain why E. coli has more than one iron-assimilation mechanism.

(ii) Aerobactin- and Enterobactin-Mediated Iron-Assimilation in S. typhimurium Infections

Using enterobactin-negative mutants of S. typhimurium, Yancey et al. (1979) showed that the production of enterobactin was a requirement for growth in serum and for high virulence in mice suggesting that the enterobactin mediated iron-assimilation mechanism was an important virulence determinant of S. typhimurium. However, recent evidence has suggested otherwise and has questioned the importance of enterobactin in vivo (Benjamin et al., 1985).

Colonna et al. (1985) have shown that plasmids isolated from epidemic Salmonella species encode for the hydroxamate-mediated iron-uptake mechanism and suggest that the aerobactin-mediated iron-assimilation system in S. typhimurium and other Salmonella species is of similar importance to the E. coli system during infection.

(b) Pathogenic Neisseria species

There are conflicting reports on whether the pathogenic Neisseria

species, N. gonorrhoeae and N. meningitidis, produce extracellular siderophores - gonobactin and meningobactin respectively (Payne & Finkelstein, 1978; Archibald & De Voe, 1980; Mickelsen & Sparling, 1981; Yancey & Finkelstein, 1981; West & Sparling, 1985).

N. gonorrhoeae does, however, express several new outer membrane proteins in response to iron limitation. The number of proteins produced is not only strain dependent, but dependent on the iron source, for example, transferrin, lactoferrin, hemin or haemoglobin. Although the number of proteins expressed varied, all strains produced a 36 kilo dalton protein with all four iron sources and this protein may play a central role in iron acquisition in N. gonorrhoeae (West & Sparling, 1985).

N. meningitidis may also obtain iron from various sources, for example, transferrin, haemoglobin, lactoferrin, myoglobin and mucin (Archibald & De Voe, 1980; Mickelsen et al., 1982). Of these sources, transferrin is perhaps the most important since this protein represents the only ready source of iron in the bloodstream. For N. meningitidis to multiply during a systemic infection it must obtain iron from this source (Simonson & De Voe, 1983).

Experimental evidence has suggested that direct contact of the protein at the bacterial surface is required for the incorporation of transferrin by N. meningitidis (Archibald & De Voe, 1979, 1980; Simonson et al., 1982) and that the production of cell free siderophores is unnecessary. It has been suggested that the organism recognises and perhaps binds the transferrin on the outer membrane and directly removes iron from the protein (Simonson & De Voe, 1983).

When grown in iron-deficient media N. meningitidis produces a 69 kilo dalton protein in the outer membrane, however a non-pathogenic Neisseria, N. flava is incapable of producing this protein and is unable

to utilise transferrin-iron strongly suggesting that this ability to acquire iron from transferrin is an important aspect of virulence in Neisseria species (Simonson & De Voe, 1983).

(c) Pathogenic *Vibrio* species

During outbreaks of cholera, classical or ElTor biotypes of *V. cholerae* can be isolated from patients and from sewage-contaminated waters (Finkelstein, 1973). *V. cholerae* ElTor strains can also be isolated in coastal waters in areas free from the disease (Kaper et al., 1979). In these cases iron is usually limiting and not freely available. When *V. cholerae* is grown in iron-depleted media a phenolate-type siderophore (vibriobactin) is produced which is similar to but not identical to enterobactin. At least six new proteins are found in the outer membrane in iron-starved cells and these iron-regulated membrane proteins are expressed by *V. cholerae* strains isolated from the intestines of infant rabbits (Payne & Finkelstein, 1978; Sigel & Payne, 1982; Sciortino & Finkelstein, 1983).

Using mutants of *V. cholerae* defective in the iron-vibriobactin transport system, the requirement of a functional siderophore-mediated iron-transport system in the pathogenicity of *V. cholerae* was ^{investigated} by Sigel et al. (1985). However the mutants retained their ability to multiply and produce disease in the infant mouse suggesting that the iron-vibriobactin transport system was not required for virulence or that in the host *V. cholerae* may obtain iron from a different source.

Vibrio vulnificus, a halophilic bacterium has been associated with a number of severe and sometimes fatal extraintestinal infections (Blake et al., 1979). Clinical and experimental evidence has suggested that iron plays an important role in the ability of *V. vulnificus* to cause infections (Wright et al., 1981). Under iron-limiting conditions

V. vulnificus can produce both hydroxamate and phenolate-type siderophores (Simpson & Oliver, 1983). However, the role, if any, of these siderophores in the virulence of V. vulnificus has not yet been determined, since the bacterium requires elevated iron levels to compete successfully with transferrin for iron in human serum and consequently produce a systemic infection (Wright et al., 1981).

Other pathogenic Vibrio species have been investigated for their ability to produce siderophores and evidence strongly suggests that several different iron-transport systems can be found in the genus Vibrio although siderophore production in V. parahaemolyticus or V. alginolyticus has not yet been demonstrated. The only Vibrio species in which a siderophore-mediated iron-uptake system has been shown to be an important virulence determinant is the marine pathogen V. anguillarum (Andrus et al., 1983).

(d) Iron-Sequestering System Encoded on a Virulence Plasmid in the Marine Pathogen V. anguillarum 775 (pJMI)

Over the past decade several workers have studied an iron-sequestering system in V. anguillarum which is encoded on a virulence plasmid, pJMI (Crosa et al., 1977; Crosa et al., 1980, 1981; Crosa & Hodges, 1981; Walter et al., 1983; Toranzo et al., 1983; Tolmasky & Crosa, 1984; Actis et al., 1985; Tolmasky et al., 1985; Actis et al., 1986; Wolf & Crosa, 1986).

In 1977, Crosa et al. isolated V. anguillarum strains from coho salmon. Analysis of several strains from different geographic origins consistently showed the presence of a 50-megadalton (Mdal) plasmid class in highly virulent strains. In one specific case, two strains isolated in Canada (V_1 and V_2), the highly virulent V_1 contained a

50-Mdal plasmid whereas V_2 appeared to have lost the plasmid giving rise to a less virulent strain. These first observations suggested that this 50-Mdal plasmid class was associated with the virulence of V. anguillarum.

To determine whether a 50 Mdal plasmid, pJM1, from V. anguillarum 775 was associated with the virulence of this strain, curing experiments were done (Crosa et al., 1980). Since no markers were present on pJM1, ampicillin resistance genes were inserted into the plasmid to monitor plasmid loss. Transposition of ampicillin resistance genes from the TnA sequence TnI in the E. coli plasmid RP₄ added an easily detectable genetic marker in pJM1. By incubating cultures of V. anguillarum 775 containing the new plasmid, pJM11 at 37°C for 48h or in the presence of ethidium bromide clones cured of plasmid were obtained. Analysis of the virulence of these cured clones in fish confirmed that plasmid loss was correlated with attenuation of virulence.

To determine if this plasmid, pJM1 was associated with an iron-sequestering system, the growth kinetics of V. anguillarum strains of high virulence and the cured, low virulence, derivatives were compared in minimal medium in the presence of transferrin (Crosa, 1980). Addition of transferrin to the culture medium inhibited the growth of low virulence, plasmid-less, cured derivatives and this was reversed by the addition of free ferric iron. Growth of strains of V. anguillarum 775 which contained plasmids pJM1 or pJM11 was not affected by the presence of transferrin. These preliminary results suggested that pJM1 specified an iron-sequestering system. In experimental infections of fish with low virulence, cured derivatives of V. anguillarum, the mean lethal dose (LD₅₀) decreased about 300-fold when iron was included in the inoculum, whereas added iron did not affect

the LD₅₀ value of V. anguillarum 775. Thus, the importance of iron, and the plasmid pJM1, in the virulence of V. anguillarum 775 was established.

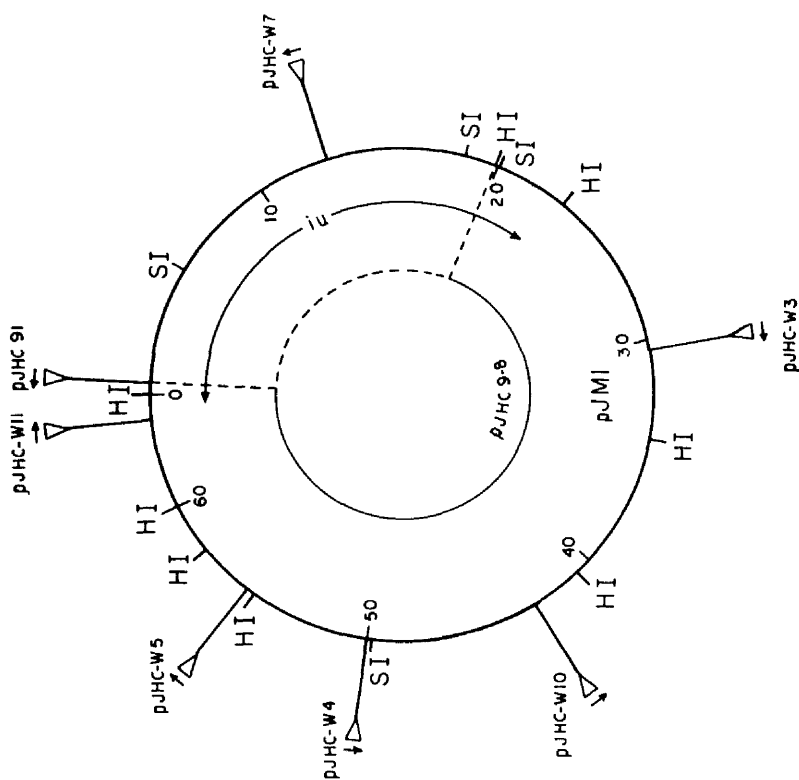
Further experiments showed that the presence of pJM1 stimulated the incorporation of radioactive iron into non-growing V. anguillarum 775 cells which also indicated the presence of an efficient plasmid-mediated iron-uptake system (Crosa & Hodges, 1981). Preliminary analysis of the outer membrane proteins synthesised by V. anguillarum 775 showed the appearance of at least two proteins under conditions of iron limitation, OM2, an 86 kilo dalton protein associated with the presence of pJM1 and OM3, a 79 kilo dalton protein.

The iron-sequestering system encoded on plasmid pJM1 was later investigated at a molecular level. Cloning experiments in which the transposon Tn1 was inserted into pJM1 at different sites localised a 20kb fragment on the plasmid which encoded two components of the iron-uptake system, a membrane band receptor (OM2) and a diffusible siderophore. Several iron-deficient derivatives were obtained. These derivatives could either synthesise OM2, but not the siderophore, or could synthesise neither the siderophore nor the membrane receptor. The two classes were distinguished by crossfeeding experiments. The first class (SID⁽⁻⁾ OM2⁽⁺⁾) were cross-fed by supernatant fluids from wild-type, plasmid-carrying strains of V. anguillarum, whereas the second class (SID⁽⁻⁾ OM2⁽⁻⁾) was not. A physical map of pJM1 based on specific cleavage by two endonucleases, BamH1 and SalI and a genetic map based on the Tn1 insertion derivatives is shown in figure 7 (Walter et al., 1983; Crosa et al., 1983).

The essential regions of the pJM1-mediated iron-uptake system were then cloned into a cosmid vector, pVK102 and introduced into low

Figure 7. Genetic and Physical Map of the pJM1 Genome.

From Walter et al. (1983). Reproduced with the
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for Microbiology.



Genetic and physical map of the pJM1 genome. The ordering of restriction endonuclease fragments was obtained by a combination of double digestions and molecular cloning of partial digests of the pJM1 plasmid. The iron uptake region is indicated by a line ending in two arrows and marked in. The ends of this region have not been determined but may possibly be somewhere in *Bam*HI fragments 5 and 6. The symbol ∇ indicates the sites of *Tn*/ insertions, and the arrow over this symbol indicates the orientation of the *Tn*/ sequence with respect to beta-lactamase gene transcription. Each different *Tn*/ insertion is identified by the initials corresponding to the plasmid generated by the insertion, for example, pJHC-W3. The deletion plasmid pJHC9-8 is also shown as a smaller circle within the pJM1 map. The dotted lines on pJHC9-8 indicate the extent of the deletion of pJM1 material. The restriction endonuclease cleavage sites are given as: B1, *Bam*HI; S1, *Sal*I. The numbers inside the map are the coordinates in kb.

virulence, iron-deficient V. anguillarum strains in conjugation experiments with the broad-range host helper plasmid pRK2013. Three recombinant clones possessed genetic determinants for receptor ability. Production of receptor activity was correlated with the presence of OM2 and two clones also coded for siderophore activity, although at a much lower level than the wild-type strain. Strains which harboured either of these clones were unable to grow under iron-limiting conditions, but this inability was overcome when other indigenous pJm1 derivatives were present, even when the indigenous plasmids possessed lesions in genes involved in siderophore activity or in both siderophore and receptor activity. This suggested that another function mediated by pJm1, possibly a trans-acting factor, could regulate the synthesis of siderophore (Tolmasky & Crosa, 1984).

Finally, to show that the receptor protein OM2 was actually encoded by the plasmid pJm1, pJm1 DNA fragments obtained by the digestion of endonucleases were cloned into cosmid vectors and transferred to E. coli. The subsequent synthesis of OM2 in E. coli proved that the OM2 protein was encoded by the pJm1 plasmid and was not of chromosomal origin (Actis et al., 1985). Membrane protein iodination experiments, together with protease treatment of whole cells indicated that the OM2 protein was exposed to the outside environment of the V. anguillarum cell.

The chemical structure of the siderophore encoded by plasmid pJm1 has recently been partially characterised by mass spectrometry, elemental analysis, resonance Raman spectrometry and infrared spectrometry (Actis et al., 1986) and has been given the name anguibactin. The siderophore was isolated from supernates of iron-deficient cultures and purified by adsorption and gel filtration columns. The resulting

neutral compound was found to have a molecular weight of 348 with a chemical formula of $C_{15}H_{16}N_4O_4S$. It appears to have a novel diphenolic structure and behaves like a catechol in colorimetric reactions. The siderophore closest in molecular properties to anguibactin was the monophenic siderophore, pyochelin, produced by Ps. aeruginosa.

Purified anguibactin exhibited specific growth-promoting activities under iron-limiting conditions for siderophore-deficient mutants of V. anguillarum 775.

The role of the siderophore during infections in fish with vibriosis was investigated using the wild-type strain, V. anguillarum 775 (pJm1) and siderophore-deficient mutants (Wolf & Crosa, 1986).

Experimental infections of salmonid fish with mixtures consisting of the wild-type strain and a siderophore-deficient, receptor-proficient mutant resulted in recovery of both wild-type and mutant strain, while in infections with mixtures consisting of the wild-type strain and a siderophore-deficient, receptor-deficient mutant, only the wild-type strain could be recovered. These results suggested that the V. anguillarum plasmid-mediated siderophore was produced in vivo in a diffusible form and that it was an important factor of virulence. However, the siderophore was not isolated directly from any fish tissue fluid.

In summary, the virulence plasmid pJm1 encodes for an iron-uptake system consisting of a diffusible siderophore, anguibactin, and an 86 kilo dalton molecular weight receptor protein OM2. This powerful iron-sequestering system appears to be an important virulence determinant of V. anguillarum 775 (pJm1) and related strains. However this is not the case for many other strains of V. anguillarum.

(e) Incidence of Plasmid pJM1 and Another Iron-Sequestering System in *V. anguillarum* Isolated From Different Geographical Origins

Although the presence of the plasmid pJM1 was correlated with high virulence in strains of *V. anguillarum* isolated from the Pacific Northwest and Canada, high virulence strains isolated in Europe at the same time did not contain the plasmid pJM1 or any similar plasmid (Crosa et al., 1980). The first recorded plasmid-mediated iron-uptake system in highly virulent strains isolated in Europe was reported by Tolmasky et al. (1985). *V. anguillarum* strains isolated from diseased trout in the Northwest of Spain harboured a plasmid that showed extensive homology with pJM1. These plasmid-containing strains produced outer membrane proteins of 79 kilo dalton and 86 kilo dalton molecular weight, the latter cross-reacting immunologically with antiserum raised against OM2 protein. A siderophore similar in activity to anguibactin was also produced. Thus the incidence of pJM1-class plasmids is not confined to one geographic source.

However, in a molecular study of factors associated with virulence of marine vibrios isolated from striped bass in Chesapeake Bay (Toranzo et al., 1983) plasmid-less, highly virulent strains of *V. anguillarum* were isolated. These strains were similar to the Northwest strains in that virulence was correlated with their ability to grow under conditions of iron limitation. Analysis of outer membranes under these conditions showed that additional proteins were induced in these strains but they were not related to the OM2 protein. These observations confirmed an earlier study by Crosa (1981) which showed that cell envelope proteins induced under conditions of iron limitation differed in different serotypes of *V. anguillarum* (see Table 7).

Table 7. Virulence-Associated Properties of *Vibrio anguillarum*

Strain	Serotype	Plasmid Content	Virulence LD ₅₀ (i)	Ability to grow in presence of Transferrin	Number and molecular weight of iron induced proteins
PT514	B	-	3.2×10^2	+	67,000 ; 76,000 ; 81,000
NCMB6	I	+ (Small plasmid)	1.2×10^4	+	72,000
2911	I	-	1.1×10^5	+	72,000 ; 66,000
1800	I	-	2.0×10^8	+	69,000 ; 72,000 79,000
775	I	+ (pJMI)	0.9×10^3	+	79,000 ; 86,000

(i) Virulence was tested on juvenile coho salmon (*Oncorhynchus kitsutch*) weighing about 14g. Virulence is quantified as LD₅₀ values. Table is adapted from Crosa (1981) and Crosa and Hodges (1981).

Several new, high molecular weight proteins were induced which were heterogeneous in size and different from OM2. These studies indicate a second iron-sequestering system which is chromosomally mediated and which is important in the virulence of plasmidless strains of V. anguillarum.

In the case of V. ordalii, no virulence plasmid encoding an iron-sequestering system has yet been shown (Schiewe & Crosa, 1981).

Objects of Research

A virulence plasmid encoding an iron-uptake system has been previously reported in strains of Vibrio anguillarum but subsequent studies have shown that virulent, plasmidless strains can also grow under iron-limiting conditions.

The primary objective of this study was to determine the relationship between plasmid carriage and ability to grow under iron-limiting conditions in Vibrio strains isolated from moribund fish or oysters. Such an investigation into the iron-uptake systems of V. anguillarum would include assaying for extracellular siderophores and the examination of cell envelopes for new membrane proteins produced under conditions of iron-limitation.

Secondly, having investigated the components of the iron-sequestering systems produced in vitro it was important to determine whether those siderophores produced in vitro were produced during infections of rainbow trout (Salmo gairdneri).

Materials and Methods

1. Bacterial Strains - Designation and Origin

Fish Pathogens	NCMB	6	<u>V. anguillarum</u>	Bagge & Bagge (1956)
		775	"	Grosa <u>et al.</u> (1977)
		636	"	(a)
		827	"	
		1197	"	
		1445	"	
		91079	"	Horne <u>et al.</u> (1977)
Bivalve Pathogens		2981	"	(b)
		4979	"	
		5679	"	
	NCMB	1336	<u>V. tubiashi</u>	Tubiash <u>et al.</u> (1965,1975)
	"	1337	"	
	"	1338	<u>Vibrio species</u>	
	"	1339	<u>V. alginolyticus</u>	
	"	1340	<u>V. tubiashi</u>	(c)
	"	2164	<u>V. anguillarum</u>	Jeffries (1982)
	"	2165	"	
	"	2166	"	
		B51	<u>Vibrio species</u>	Garland <u>et al.</u> (1983)
		B55	"	
		B2	"	
		T61	"	
		B1	"	

- (a) Isolated from moribund salmonid fish in Scandinavia. Obtained from Dr. M. Horne, University of Stirling.
- (b) Obtained from Dr. B. Austin, Heriot-Watt University.
- (c) Obtained from the National Collection of Marine Bacteria, Torry, Aberdeen.

2. Media

Various media were used throughout for measurement of bacterial growth, plasmid analysis, cell envelope preparations, radioactive iron (^{55}Fe)-uptake assays and siderophore production. Their composition is in appendix 1.

3. Maintenance of Cultures

The organisms, which were originally taken from freeze-dried ampoules, were reconstituted in NBS (see appendix 1) and plated onto NAS (see appendix 1). Cultures were maintained on NAS slopes and passaged on fresh NAS slopes monthly with routine gram staining to confirm culture purity.

4. Measurement of Bacterial Growth

The organisms were grown in 50 ml NBS, NBST or NBST plus FeCl_3 (see appendix 1) in a 250ml dimpled, conical flask and incubated at 25°C on an orbital shaker operating at 150rpm. Growth was estimated by measuring the $A_{600\text{nm}}$ of samples withdrawn at 1h intervals; when $A_{600\text{nm}}$ exceeded 1.00 the culture was diluted with the appropriate medium to bring the adsorption value to within the range of 0.50-1.00. Adsorption values were measured on an SP8-100 Ultraviolet Spectrophotometer (Pye, Unicam). The appropriate uninoculated medium served as a blank.

5. Biochemical Assays

(a) Protein Estimation

Protein estimation was carried out by the method of Bradford (1976) with bovine serum albumin (BSA) (Sigma) as standard.

(b) Estimation of Ferric Iron Present in Culture Medium

The amount of iron present in NBS, VMM and TSM (see appendix 1) was calculated using "The Iron Binding Capacity Kit" (Sigma). Using the above kit the amount of human transferrin (Sigma) required to bind the available iron (Fe^{3+}) was also calculated.

The required amount of human transferrin was calculated as follows:

One molecule of transferrin is capable of binding two molecules of iron (Bullen et al., 1978), ie, one mole of transferrin (80,000g) can bind two moles of Fe^{3+} (111.6g). If the amount of free iron available is Xg/ml, the amount of transferrin required (in g/ml) to bind the iron would be,

$$716.846(X) \text{ g/ml}.$$

(c) Assays for Detection of Phenolate- and Hydroxamate type Siderophores

(i) The Arnow Assay For Phenolate-Type Siderophores

Phenolate-type siderophores were detected by the colorimetric assay of Arnow (1937). To 1ml of bacterial culture supernate or lyophilised culture supernate resuspended to $\frac{1}{10}$ of the original volume, the following were added sequentially: 1ml 0.5N hydrochloric acid, 1ml nitrite molybdate reagent (10g sodium nitrite and 10g sodium molybdate in 100ml distilled water), 1ml 1N sodium hydroxide and 1ml distilled water. After each addition the samples were mixed thoroughly. The positive red colour was measured at 515nm in a Pye Unicam SP8-100 ultraviolet spectrophotometer. The minimum amount which can be detected with this assay is 5nmol of diphenol (Barnum, 1977). 3,4-dihydroxybenzoic acid (Sigma) was used as a positive control and fresh culture medium as a blank.

(ii) The Ferric Perchlorate Test for Hydroxamate-Type Siderophores

Hydroxamate siderophores were detected by the addition of 1ml of a solution of ferric perchlorate (2.5 mg/ml) and perchloric acid (0.14 g/ml) to 1ml culture supernate or lyophilised culture supernate resuspended to $\frac{1}{10}$ of the original volume. A red/brown colour was indicative of a positive hydroxamate reaction (Atkin et al., 1970). Aerobactin (kindly supplied by E. Griffiths, London) was used as a positive hydroxamate standard and fresh culture medium as a negative control.

(iii) Csaky Test for Hydroxamate-Type Siderophores

This was a modification of the original Csaky Test (Csaky, 1948). To 0.5ml bacterial culture supernate, 0.5ml 6M sulphuric acid was added and the sample was hydrolysed in a sealed tube by autoclaving at 121°C for 18h. Sodium acetate solution (35% w/v) was added to the hydrolysed sample to adjust the pH to 5.5. After the sequential addition of 1ml sulphanilic acid solution (1% w/v in 30% acetic acid) and 0.5ml iodine solution (1.3% w/v in 30% acetic acid), the solution was mixed and allowed to stand for 5 minutes at room temperature. To decolourise the solution, 0.5ml sodium thiosulphate solution (2.5% w/v) was added. After mixing the solutions, 0.5ml 3N HCl and 1ml of α -naphthylamine solution (0.3% w/v in 30% acetic acid) (Sigma) was added and the volume adjusted to 10ml with distilled water. The pink colour, indicative of a positive reaction, was allowed to develop for 30 minutes at room temperature. Aerobactin was used as a positive hydroxamate standard and fresh culture medium as a negative control. With all samples, the assay was carried out with and without the hydrolysis step.

(d) Radioactive Iron-(^{55}Fe)-Uptake Assays

This assay was modified from that described by Crosa and Hodges (1981). The bacteria were grown overnight in 10ml VMMT (see appendix 1) in a 100ml conical flask at 25°C on an orbital shaker operating at 150rpm and the cultures were harvested by centrifugation at 12,000g for 5 minutes at room temperature. The bacteria were then washed and resuspended in fresh medium to a density of 10^8 cells ml⁻¹. After incubation, as before, for 4h, to deplete intracellular pools of iron, the bacteria were collected by centrifugation as above and resuspended to a density of 10^7 cells ml⁻¹ in VMM (see appendix 1) supplemented with carrier free (^{55}Fe)Cl₃ (Amersham International plc) (Specific activity 5.2 MBq/μg ^{55}Fe ; 37 KBq/ml). The cultures were shaken at 25°C on an orbital shaker operating at 150rpm and 1ml samples were removed at 4 minute intervals and filtered through 0.45μm mean pore diameter nitrocellulose filters (Millipore). The filters were washed with 100mM sodium citrate, dried and the radioactivity counted in a liquid scintillation spectrometer (Packard Tri-Carb model 300C) after dissolution of samples in a toluene-based scintillation fluid (Ecoscint, National Diagnostics).

(e) Inhibition of Uptake of Radioactive Iron (^{55}Fe)

(i) The above assay (see section 5(d)) was modified as follows: after incubation of the bacteria in VMMT for 4h at 25°C, the cells were recovered by centrifugation at 12,000g and resuspended in 3-day culture supernates of *Vibrio* strains grown in VMMT, TSM or TSMT (see appendix 1). Carrier free (^{55}Fe)Cl₃ was added to a final concentration of 37 KBq/ml and a 1ml sample was taken after 6 minutes.

(ii) By Aerobactin and Desferal

Again the assay was modified as follows: after incubation of the

bacteria in VMMT for 4h at 25°C, the cells were recovered by centrifugation at 12,000g and resuspended in the respective 3-day culture supernate grown in NBST, supplemented with either desferal (Ciba-Geigy) or aerobactin to a final concentration of 0.5 mg/ml. Carrier-free (^{55}Fe)Cl₃ was added to a final concentration of 37 KBq/ml and a 1ml sample was taken after 6 minutes.

6. Plasmid Analysis

(a) Mini-Plasmid Preparations

(i) The Kado and Liu Method

This method was modified from that of Kado and Liu (1981). The bacterial cells were grown in 10ml NBS (see appendix I) in a 100ml conical flask and incubated at 25°C on an orbital shaker operating at 150rpm until the late exponential phase of growth. The cells (1.5ml) were collected by centrifugation at 12,000g for 5 minutes at room temperature. The pellet was resuspended in 100µl of lysis buffer (see appendix II) and incubated at 60°C for 45 minutes. The plasmid DNA was extracted with 100µl of phenol:chloroform mixture (1:1 ratio v/v). The emulsion was separated by centrifugation at 12,000g for 10 minutes at room temperature and the top, aqueous layer retained and stored at -20°C.

(ii) The Holmes and Quigley Method

This method was adapted from the procedure of Holmes and Quigley (1981). A loopful of bacteria from an overnight (25°C) culture on NAS (see appendix I) was transferred to an Eppendorf tube and resuspended in 100µl of STET buffer (see appendix II). To the suspension, 8µl lysozyme solution (10 µg/ml in distilled water) was added and the solution incubated for 5 minutes at room temperature.

The sample was boiled for 40 seconds and centrifuged at 12,000g for 10 minutes. The supernate was transferred to a clean Eppendorf tube and the final volume adjusted to 200 μ l with deionised, distilled water. An equal volume of water-saturated phenol was added and, after thorough mixing, the emulsion was separated by centrifugation at 12,000g for 5 minutes at room temperature; 200 μ l of deionised distilled water was then added to the aqueous layer and the phenol extraction step was repeated. To the aqueous layer 400 μ l of chloroform was added, the sample centrifuged as before and the aqueous layer retained. The chloroform step was repeated. To the final aqueous sample, 10 μ l of 3M sodium acetate and 250 μ l of absolute alcohol were added. The plasmid DNA was allowed to precipitate for 1h at -70°C or overnight at -20°C and the DNA pellet was collected by centrifugation at 12,000g for 10 minutes. The pellet was washed by centrifugation in 70% ethanol, dried, resuspended in 20 μ l of TE buffer (see appendix II) and stored at -20°C.

(b) Electrophoresis of Plasmid DNA

(i) Preparation of Agarose Gel

For electrophoresis of plasmid DNA, 0.7% agarose (Type 1: LOW EEO; Sigma) in the appropriate electrophoresis buffer (see appendix II) was melted in a microwave oven then poured onto a glass plate (26cm x 12.6cm) to approximately 5mm thickness. To 15 μ l of sample, 5 μ l of tracking dye (see appendix II) was added and 10 μ l was then added directly to wells in the gel.

(ii) Electrophoresis of Sample

Electrophoresis was carried out with a Shandon SAE power unit at 25V, overnight at room temperature with the appropriate electro-

phoresis buffer (see appendix II).

(iii) Analysis of Agarose Gel

After electrophoresis the gel was stained with ethidium bromide (0.5 µg/ml) for 20 minutes at room temperature, and then washed with distilled water for 1h. Gels were visualised with a short-range ultraviolet UVP chromato-vue cabinet transilluminator.

7. SDS-Polyacrylamide Gel Electrophoresis of Proteins and LPS

(a) Preparation of Polyacrylamide Gels and Electrophoresis of Samples

(i) Preparation of Vertical Slab Gels

The method was based on those described by Laemmli (1970) and Ames (1974) with a vertical slab gel tank to a design similar to the Studier-type slab gel apparatus (Studier, 1973). The stock solutions and the recipe for gel preparation are shown in appendix III. Separating (lower) and stacking (upper) gels contained 12.5% or 10% (w/v) and 4.5% (w/v) acrylamide respectively. The gel was formed between two glass plates (17cm x 19cm x 0.3cm) with a spacer (1.5mm thick) running down each vertical side of the plates.

(ii) Sample Preparations

Cell envelope preparations were adjusted to 0.25-0.75 mg/ml protein as determined by the method of Bradford (1976), whereas the whole cell samples and LPS fractions were undiluted. Each sample was added to an equal volume of appropriate solubilising buffer (see appendix III) and heated to 100°C for 3 minutes prior to the addition of 20µl of sample to the gel.

(iii) Electrophoresis of Sample

Electrophoresis was performed at room temperature at a constant

current of 30mA until the tracking dye had reached the bottom of the gel. A Shandon VoKam SAE2761 power unit was used.

(b) Analysis of Gels After Electrophoresis

(i) Coomassie Blue Stain

Staining and destaining of the gels was carried out using the method of Weber and Osborn (1969). Coomassie Blue stain (see appendix III), using Coomassie Blue R250 (BDH), was used for protein staining. Gel slabs were stained overnight at room temperature and destained the following day using several changes of destaining solution (see appendix III). The gel was soaked in distilled water and stored in a heat-sealed cellophane bag.

(ii) Molecular Weight Estimation

The method used was based on those of Ornstein (1964) and Weber and Osborn (1969). When denatured by heating in the presence of excess SDS and a thiol reagent (in this case 2-mercaptoethanol), most polypeptides bind SDS in a constant weight ratio such that they have essentially identical charge densities and migrate in polyacrylamide gels of the correct porosity according to polypeptide size. Under these conditions, a plot of \log_{10} polypeptide molecular weight versus relative mobility (R_f) reveals a straight line relationship. The relative mobility refers to the mobility of the protein of interest measured with reference to the marker protein or to a tracking dye where:

$$R_f = \frac{\text{distance migrated by Protein}}{\text{distance migrated by dye.}}$$

The approach was therefore to electrophorese a set of marker polypeptides of known molecular weight and use the distance migrated by

each to construct a standard curve from which the molecular weight of the sample polypeptides could be calculated based on their mobility under the same electrophoretic conditions.

Whenever analytical SDS-PAGE was used, a mixture of polypeptides of known molecular weight was added to one track of the gel. The molecular weight marker set, SDS-7 Kit for molecular weights (Sigma), was used at all times. This contained a mixture of the following seven proteins:

α -lactalbumin (14,200 daltons)

Trypsin Inhibitor (20,100 daltons)

Trypsinogen (24,000 daltons)

Glyceraldehyde-3-Phosphate Dehydrogenase (36,000 daltons)

Egg Albumin (45,000 daltons)

Bovine Albumin (66,000 daltons)

(iii) Ferene S Stain for Iron Binding Proteins

This staining procedure was adapted from the method of Ching-Ming (1985). Ferene S stain (see appendix III) using Ferene S (3-(2 pyridyl-5,6,Bis(2-5-Furysulphonic acid)-1,2,4-triazine disodium salt) (Sigma) as the dye. Gel slabs were stained in a 1% (w/v) aqueous solution of FeCl_3 for 10 minutes at room temperature and destained overnight in destaining solution (see appendix III) at room temperature. The destaining solution was replaced by Ferene S stain until the desired level of staining had been reached, before destaining again as above. Transferrin (70 $\mu\text{g}/\text{ml}$) was used as a positive standard control.

(iv) Silver Staining for Lipopolysaccharide

This method was adapted and modified from the procedures of Oakley et al. (1980) and Tsai and Frasch (1982).

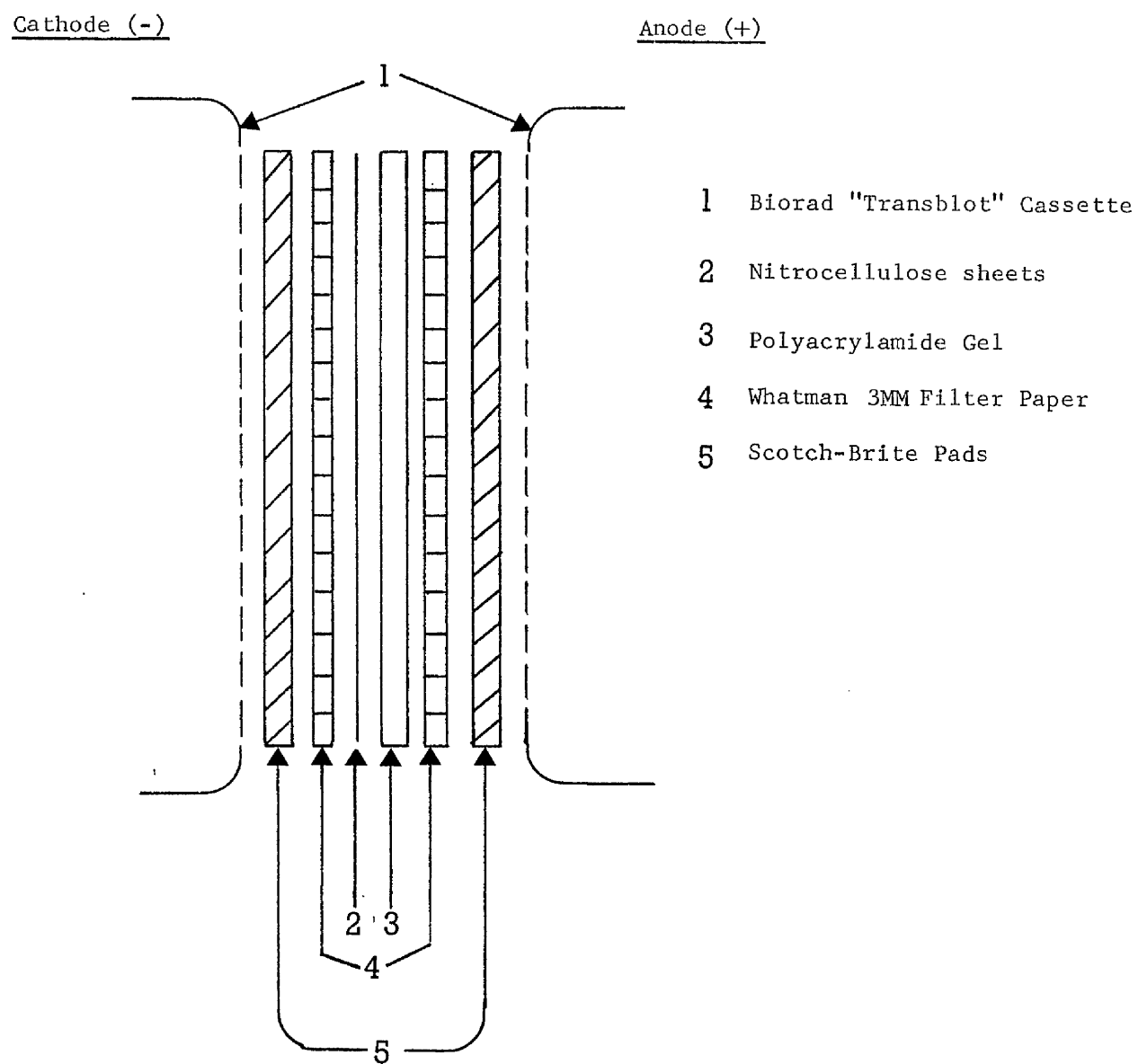
The silver stain uses silver nitrate as the key component which can bind sugars found in LPS. The LPS was fixed by incubating the slab gel in a fixing solution of ethanol:acetic acid:water (40:5:55 (v/v)) overnight at room temperature. The fixing solution was replaced with a 0.7% (w/v) periodic acid solution in ethanol:acetic acid:water (40:5:55 (v/v)) for 5 minutes at room temperature with constant shaking. The slab gel was washed with 500ml distilled water for 15 minutes with 3 changes of distilled water, 150ml freshly prepared staining reagent (see appendix III) was added and after 10 minutes replaced with distilled water, as above. The gel was then rinsed rapidly in developer (50 μ l of 37% formaldehyde in 100ml of 3% (w/v) sodium carbonate solution) and soaked in $\frac{1}{3}$ volume of developer until desired level of staining was reached. To the developer, 5ml of 2.3M citric acid was added and after 10 minutes this solution was replaced by distilled water. To preserve the gel it was soaked in 0.03% (w/v) sodium carbonate for 10 minutes and heat-sealed in a cellophane bag.

(c) Immunological Identification of Proteins and LPS

(i) Protein and LPS Blotting onto Nitrocellulose Membranes

Electroblotting was carried out by the method of Towbin et al. (1979) in a Bio-rad "Transblot" transfer apparatus. Diagrammatic presentation of the slab gel and nitrocellulose membrane assemblage is shown in figure 8. The slab gel, nitrocellulose membrane (Hybond-C, Amersham) and filter paper (3MM, Whatman) were soaked in precooled transfer buffer (see appendix III), the cassette assembled and placed into an electrophoretic transfer chamber containing 2.5 litres of precooled transfer buffer with the nitrocellulose membrane facing the cathode. Electrophoretic transfer was accomplished at 220mA in 3h at

Figure 8. Assemblage of Electroblotting Cassette Apparatus.



(Assembly parts are shown separated for visualisation only).

Adapted from Towbin et al. (1979).

room temperature using a Shandon 50V 1A power unit with a cooling supply within the transfer chamber. After transfer, the slab gel was stained with either coomassie blue protein stain or silver stain for LPS.

(ii) Immunological Detection of Protein and LPS on Nitrocellulose Membranes

Immediately following transfer, the nitrocellulose sheet was immersed in wash buffer (see appendix III) containing 5% gelatin (Oxoid) or 5% horse serum (Oxoid) and incubated at 42°C for 45 minutes. The sheet was transferred to fresh 0.5% gelatin or 0.5% horse serum in wash buffer containing the appropriate volume of antiserum (100-200µl) and incubated at 4°C for 16-18h. The nitrocellulose sheet was then washed with shaking for 4h in 8 changes of 100ml wash buffer at room temperature and immersed in fresh wash buffer containing $1/3,000$ dilution of sheep-anti-rabbit horseradish peroxidase conjugate (HRP) (Scottish Antibody Production Unit) and incubated for 1½h at 37°C. The nitrocellulose sheet was removed and washed as described above. The substrate, 4-chloro-naphthol (BioRad) (see appendix III), was then added until the desired level of staining had been reached. The nitrocellulose sheet was finally washed in distilled water, air dried and stored.

(d) Detection of ⁵⁹Fe-Labelled Proteins from Whole Cell Preparations

Bacterial cells were grown in 50ml NBS or NBST (see appendix 1) in 250ml dimpled, conical flasks overnight at 25°C on an orbital shaker operating at 150rpm. To 10ml bacterial culture 100µCi ⁵⁹FeCl₃ (Amersham) (Specific activity 530 MBq/mgFe) was added and the cultures incubated as before for 1h at 25°C. Bacterial suspensions (3ml) were centrifuged at 12,000g for 5 minutes at room temperature in

Eppendorf tubes and bacteria resuspended in 1.5ml 0.46M Tris/HCl pH 7.8. The centrifugation step was repeated and the pellet was finally resuspended in 1.0ml 0.46M Tris/HCl pH 7.8, 0.5ml solubilising buffer (see appendix III) added and the mixture heated to 100°C for 3 minutes before 80µl of sample was loaded onto a 12.5% polyacrylamide gel. After electrophoresis, the gel was stained using Coomassie blue stain, destained (see appendix III and Section 7.b.(i)) and washed in distilled water. The gel was dried using a Savant slab gel dryer Model SGD-200 and placed against LKB Ultrafilm for 4 weeks at -70°C.

The Ultrafilm was allowed to warm from -70°C to room temperature and placed in developer (Kodak, DX-80) for 5 minutes. After this time the film was transferred to 3% (v/v) acetic acid for 30 seconds and finally transferred to fixer solution (Kodak, Unifix) for a further 5 minutes. The film was washed in cold tap water for 10-15 minutes and dried.

8. Preparation of Cell Envelope Fractions and Whole Cell Samples

(a) Preparation of Cell Envelope Fractions

(i) Chemical Lysis and X-Press Method

The following procedure was adapted from the methods of Yamato et al. (1975) and Owen et al. (1982).

The bacterial cells were grown in 500ml NBS or NBST (see appendix I) in 2 litre dimpled, conical flasks and incubated for 24h at 25°C on an orbital shaker operating at 150rpm. The cells were harvested by centrifugation at 12,000g for 15 minutes at 4°C. The bacteria were resuspended in cold buffer (see appendix IV) and kept at 4°C. The centrifugation step was repeated and the cell pellet resuspended to $1/100$

volume of original culture in 0.04M Tris pH 8.1, containing 25% (w/v) sucrose and kept at 4°C. The cells and all solutions were kept in ice at all times and the following solutions were added sequentially:

- (a) 0.5ml lysozyme solution (770 µg/ml in 0.25M Tris/HCl pH 8.1)
- (b) 0.5ml EDTA solution (20 mg/ml in distilled water)
- (c) 0.75ml Brij 58 solution (5% w/v) in 0.01M Tris/HCl pH 8.1)
- (d) 0.75ml 0.1M magnesium sulphate solution
- (e) bovine pancreas deoxyribonuclease I (DNase I, Sigma, 0.1% (w/v)

in 0.15M NaCl pH 5.0) to a final concentration of 3 µg/ml.

The cell suspension was kept overnight at 4°C and disrupted in a pre-chilled X-Press (Biox X-Press Cell Disintegrator). An equal volume of 3mM EDTA (pH 7.2) was added and the suspension was centrifuged at 78,000g for 3h at 4°C. The pellet was resuspended in 10% (w/v) sucrose in 3mM EDTA pH 7.2 at 4°C and centrifuged at 12,000g at 4°C for 5 minutes to remove any remaining whole cells. The supernate was retained and stored at -20°C.

(ii) EDTA Extraction Procedure

The procedure was adapted from the method of Zollinger et al. (1972) Bacterial cells were grown and collected as previously described (see section 8.a.(i)) and resuspended in EDTA extraction buffer (see appendix IV) to a final volume of 50ml. The suspension was incubated at 60°C for 30 minutes and then subjected to mild shear by twice passing it through a 1½ inch, 23 gauge hypodermic needle by manual pressure. Undisrupted cells were pelleted by centrifugation at 12,000g for 10 minutes at 4°C. The supernate was centrifuged at 84,000g for 2h at 4°C and then allowed to stand overnight at 4°C. A small opalescent pellet settled at the bottom of the centrifuge tube and was retained.

This preparation was washed in sterile distilled water, collected by high-speed centrifugation as before and stored at -20°C .

(b) Preparation of Whole Cell Samples for Polyacrylamide Electrophoresis

The bacterial cells were grown in NBS or NBST (see appendix 1) in 250ml dimpled, conical flasks and incubated overnight at 25°C on an orbital shaker operating at 150rpm. The cells were collected by centrifugation at 12,000g for 5 minutes at 4°C , washed twice in 0.46M Tris/HCl pH 7.8 and finally resuspended in 0.46M Tris/HCl pH 7.8 to give an adsorption value of 0.1 at 600nm (Pye Unicam SP-550 Ultraviolet Spectrophotometer). To 1ml of the standardized bacterial suspension, 0.5ml solubilising buffer (see appendix III) was added and the solution heated to 100°C for 3 minutes prior to loading 20 μl of the sample on to a prepared polyacrylamide gel.

9. Lipopolysaccharide Extraction

The method was based on the procedure of Westphäl (Westphäl *et al.*, 1952; Keleti & Lederer, 1974). The bacteria were grown in 100ml NBS (see appendix 1) in 2 litre dimpled, conical flasks and incubated overnight at 25°C on an orbital shaker operating at 150rpm. Cells were collected by centrifugation at 12,000g for 15 minutes at 4°C , resuspended in 10ml hot, sterile, distilled water (68°C) and 10ml of 90% (w/v) phenol, also preheated to 68°C and incubated for 30 minutes at 68°C . The mixture was then cooled to 10°C and centrifuged at 1,500g for 45 minutes at room temperature. The lower, phenol layer was retained. To collect LPS from the phenol layer, 4 volumes of methanol were added and a precipitate allowed to form overnight at 4°C . The precipitate was pelleted by centrifugation at 7,000g for 15 minutes

at 4°C, resuspended in distilled water and dialysed in cold, running tap water for two days followed by 3 changes of 2 litres distilled water for 24h at 4°C. The remaining suspension was lyophilised and stored at 4°C.

10. Two-Dimensional Immunoelectrophoresis

The following method was adapted from that described by Owen and Salton (1975) and Smyth et al. (1978). The preparation of cell envelope samples and antiserum used throughout these experiments were described in sections 8 and 11 respectively.

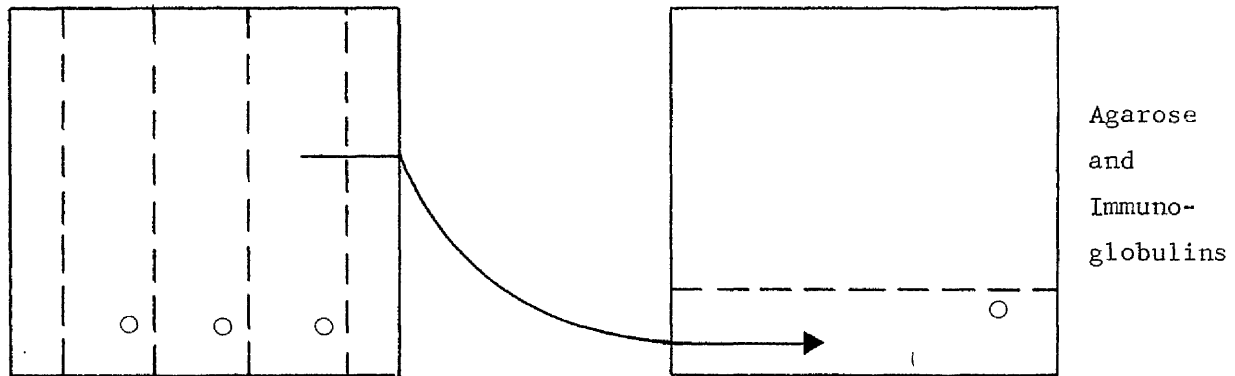
(a) Preparation of Slides for Electrophoresis

Barbital-HCl buffer (see appendix IV) containing 1% (v/v) Triton-X-100 was used throughout as the electrophoresis running buffer and was incorporated into all gels. In all experiments 1% (w/v) agarose (Type 1: low EEO, Sigma) was used; it was melted in a microwave oven and cast on glass plates (5cm x 5cm) to give a volume to surface area ratio of 0.14 ml/cm.

(b) Electrophoresis of Cell Envelope Samples

Cell envelope preparations were applied to wells of 3mm diameter (see figure 9) and the first direction electrophoresis was carried out at 40V for 90 minutes in an LKB 21M Multiphor electrophoresis tank with an Electrophoresis Constant Power supply ECPS 3000/150 (Pharmacia). An agarose strip (12mm x 50mm) containing the antigens subjected to electrophoresis was retained and the rest of the gel was replaced with an adjacent gel containing 400µl of anti-envelope immunoglobulins. Electrophoresis in the second direction was then performed at 30V overnight at 4°C.

Figure 9. Templates for preparation of Slides for Crossed Immuno-
electrophoresis



1st Dimension electrophoresis.

2nd dimension electrophoresis.

(c) Analysis of Gels After Electrophoresis

The gels were washed twice in sterile saline (1% (w/v) NaCl) and finally in distilled water before being pressed, air dried and stained with saturated nigrosine (I.C.I.) in 3% (v/v) acetic acid. The gels were destained in 3% (v/v) acetic acid until the precipitin lines could be clearly seen, washed in distilled water, air dried and kept as a permanent record.

11. Preparation of Antiserum

(a) Collection and Analysis of Antiserum

Antisera were raised in New Zealand white rabbits (2 rabbits per strain) against cell envelope fractions of V. anguillarum strains 775 and NCMB6, grown in NBST (appendix 1). An initial intramuscular injection of antigen in complete Freund's adjuvant (50:50; all subsequent intramuscular injections were in incomplete adjuvant) was followed by two further injections 7 and 14 days after the initial injection. Test blood samples (20ml) were taken on days 21, 28 and 35. Intravenous booster injections of antigen in saline (1% (w/v) NaCl) on days 47, 49 and 52 with further trial bleeds on days 56, 63 and 70 resulted in a strong polyvalent antiserum. The high levels of antibody against the antigens were maintained by subsequent intravenous booster injections every 2-3 months. Samples of antiserum were collected after day 70 as required.

(b) Collection of Serum

Serum was separated from the clot of a 20 ml blood sample and centrifuged at 5,000g for 10 minutes at room temperature to remove any remaining red blood cells. Sodium azide (1-2 grains) was added as a

preservative and the serum was stored at either 4°C or -20°C.

(c) Analysis of Test Serum

(i) Ouchterlony Gel Diffusion Test

This procedure was adapted from the method first described by Ouchterlony (1958). In each test 1% (w/v) agarose (Type 1:Low EEO, Sigma) was used in all gels. The agarose was melted in 2ml barbital-HCl buffer containing 1% (v/v) Triton-X-100 (see appendix IV) using a microwave oven and cast on glass slides (76mm x 26mm).

Samples were applied to wells of 3mm diameter and the slides were incubated in a moist atmosphere at 37°C overnight to allow the formation of precipitin lines. The slides were washed in sterile saline (1% (w/v) NaCl) at 4°C with 4 changes of saline, washed in sterile distilled water, pressed, air dried and stained with saturated nigrosine (I.C.I.) in 3% (v/v) acetic acid for 10 seconds. After destaining in 3% (v/v) acetic acid until the precipitin lines were clearly visible, the slides were washed in sterile distilled water, air dried and kept as permanent records.

(ii) Antigen-Antibody Agglutination Titres

To wells 2-11 in a row of a microtitre plate (Microtiter System), 50µl of sterile saline (1% (w/v) NaCl) was added. To well 12, 50µl of saline was added. A volume of 100µl of serum was added to well 1 and the serum was diluted in a series of two fold dilutions from well 1-11 by transferring 50µl from each well. To each well 50µl of the appropriate antigen was added, the tray shaken and incubated for 1h at 37°C. The titre of the antiserum was then read as the reciprocal of the highest dilution to give a positive agglutination reaction.

(d) Absorption of Antiserum

(i) Absorption of Antiserum with Cell Envelope Fractions

To 5ml of antiserum which had been heated to 56°C for 30 minutes, 5ml of a cell envelope fraction (5 mg/ml protein content determined by the Bradford Assay) of the appropriate bacterial strain grown in NBS (see appendix 1) was added and shaken at 150 oscillations per minute in an orbital shaker at 4°C for 30 minutes. The antibody-antigen precipitate and remaining cell envelope fractions were removed by centrifugation at 84,000g for 2h at 4°C and the supernate (absorbed antiserum) was retained.

The above procedure was repeated until there was no agglutination between antiserum and antigen and there was no antigen-antibody precipitate in the Ouchterlony gel diffusion test.

(ii) Absorption of Antiserum with Crude LPS

The above procedure (see section 11.c.(i)) was modified as follows: after incubation of antiserum at 56°C for 30 minutes an equal volume of crude LPS prepared from a 500ml culture of appropriate bacterial strain was added, and the absorbed antiserum was collected as previously stated.

(e) Ammonium Sulphate Precipitation of Serum

Sera from consecutive bleedings were pooled and immunoglobulins were partially purified by precipitation with ammonium sulphate and dialysis against sodium acetate buffer pH 5.0 (see appendix IV).

To 1 volume of serum, 2 volumes of saturated $(\text{NH}_4)_2\text{SO}_4$ was added and the mixture stirred for 1h at 4°C. The precipitate was recovered by centrifugation at 7,000g for 10 minutes at 4°C and the supernate was discarded. The pellet was resuspended in 35% saturated $(\text{NH}_4)_2\text{SO}_4$

solution and stirred for 1h at 4°C. The centrifugation step was repeated, the pellet was resuspended in sodium acetate buffer pH 5.0 (see appendix IV) and dialysed in 3 litres of this buffer with 4 changes of buffer over 24h. The partially purified immunoglobulins were then concentrated to $\frac{1}{10}$ the original volume of serum with Aquacide 11 (Calbiochem).

12. Paper Chromatography

(a) Preparation of Samples for Analysis

The following methods for the preparation of samples for both hydroxamate- and phenolate-type siderophores were adapted from those of Andrus et al. (1983) and Rogers (1973).

For hydroxamate-type siderophores, lyophilised culture supernate of bacterial cells grown in iron-limiting media (NBS-T, NBS plus desferal, TSM; see appendix 1) for 72h at 25°C on an orbital shaker operating at 150rpm, was resuspended to $\frac{1}{10}$ the original volume in sterile distilled water and applied directly to the chromatography paper.

For phenolate-type siderophores, the lyophilised culture supernate was again resuspended to $\frac{1}{10}$ the original volume in sterile distilled water. The phenolate compounds were extracted using ethyl acetate by the following procedure: to 5ml of lyophilised supernate, 5ml 0.5N HCl was added to adjust the pH to 1.5, 5ml ethyl acetate was then added and the resulting emulsion was shaken and allowed to stand for 2h. The aqueous layer and ethyl acetate layer were separated by centrifugation at 3,000g for 20 minutes at room temperature and the ethyl acetate layer retained. This layer was evaporated to dryness with a stream of nitrogen gas and finally redissolved in 0.5ml ethanol.

(b) Paper Chromatography Analysis for the Detection of Phenolate-Type Siderophores

The method was taken from the procedure of Rogers (1973). The samples, after extraction with ethyl acetate, were spotted on to Whatman No. 1 chromatography paper and ascending chromatography was carried out at room temperature for 4h using a Shandon Tank with 5% (w/v) ammonium formate plus 0.5% (v/v) formic acid as solvent. The papers were air dried and analysed (see section 12.d).

(c) Paper Chromatography Analysis for the Detection of Hydroxamate-Type Siderophores

This method was adapted from the procedure of Gibson and Magrath (1969). The samples were spotted onto Whatman No. 1 chromatography paper and ascending chromatography was carried out overnight at room temperature using a Shandon Chromatography tank with n-butanol-water-acetic acid (60:15:25 v/v) as solvent. The papers were air dried and examined as described in section 12.d.

(d) The Analysis of Chromatography Papers for the Presence of Siderophores

The dried papers were firstly examined under short-wave ultraviolet light using a UVP chromato-vue cabinet transilluminator to detect fluorescent spots. The iron-binding compounds were then detected by spraying with 1% FeCl_3 (w/v) and air drying the papers in the presence of ammonia vapour.

For the detection of phenolate compounds, 3,4-dihydroxybenzoic acid (Sigma) was used as a positive control. Aerobactin or desferal were used as positive hydroxamate-type siderophore controls.

To compare the spots which appeared on the stained papers, the

R_f value was calculated for each spot. The R_f value was defined as follows:

$$R_f = \frac{\text{Distance travelled by the Iron-Binding Compound (cm)}}{\text{Distance travelled by the solvent front (cm)}}$$

13. Experimental Fish Infections

(a) Environmental Conditions for Experimental Infections

Rainbow trout (16-19cm length), obtained from West of Scotland Trout Farm, Scotland, were maintained in tanks of 88cm x 57cm x 57cm dimensions in copper-free water at a temperature of 15-17°C. Before the commencement of experiments the fish were allowed to acclimatise for at least 4 days and were fed on a diet of Tetrafood sticks (Tetraman) before or during infection. To reduce fouling in the tanks the water was filtered through an Eheim Universal Inner Filter 2009 (Eheim Ltd.).

After the completion of each experiment all tanks were cleaned with a 2.5% solution of Dettol (Reckitt and Coleman) and washed with copper-free water for 2 days.

(b) Infection of Fish

The rainbow trout were inoculated intramuscularly at the base of the dorsal fin with 0.1ml bacterial suspension in sterile saline (3% (w/v) NaCl) ranging in concentrations from 10^{10} - 10^3 cells ml⁻¹. The bacteria were grown in 10ml NBS (see appendix 1) in 100ml conical flasks and incubated overnight at 25°C on an orbital shaker operating at 150rpm and collected by centrifugation at 12,000g for 10 minutes at 4°C. The number of cells per ml in the original culture was determined using an Improved Neubauer Counting Chamber (Weber Scientific International Ltd.). Fish were killed when showing signs of acute vibriosis, immediately frozen and stored at -20°C. Once thawed, the fish were

dissected and kidney, spleen and blood samples were removed for analysis.

(i) Tissue Extracts

The entire kidney and spleen from each fish were individually homogenised in 2ml sterile saline (3% (w/v) NaCl) in a sterile glass/teflon tissue homogenizer (Jencons) and used for bacterial examination and detection of siderophores.

(ii) Bacterial Examination

Blood and tissue homogenate samples were analysed by culturing on NAS and thiosulphate/citrate/bile salts agar (TCBS) (see appendix 1). Bacterial colonies were tested for purity and further experiments were only done when V. anguillarum was isolated in pure culture. The total number of bacteria recovered from the kidney and spleen samples were calculated by carrying out tenfold serial dilutions (10^{-1} - 10^{-6}) in sterile 3% (w/v) saline and plating 0.1ml of each dilution onto NAS and TCBS. Samples from an uninfected fish were treated in a similar manner.

(c) Detection of Bacterial Siderophores

The entire kidney and spleen homogenates were centrifuged at 12,000g for 10 minutes at 4°C to remove tissue debris and bacterial cells and the supernates retained for detection of bacterial siderophores. Samples from uninfected fish were treated in a similar manner.

(i) Detection of Phenolate-Type Siderophores

The homogenate supernates were extracted by the sequential addition of 0.5 volume of 0.5N HCl and 0.5 volume of ethyl acetate. The resulting emulsion was shaken and allowed to settle for 1-2h. The aqueous layer and ethyl acetate layer were separated by centrifugation as previously described (see section 12.a) and the ethyl acetate layer was retained and evaporated to dryness over nitrogen gas. The sample was finally redissolved in 0.05-0.1ml ethanol and applied to chromatography paper.

(ii) Detection of Hydroxamate-Type Siderophores

The homogenate samples were lyophilised, redissolved in 0.1ml sterile distilled water and applied directly to chromatography paper.

(iii) Paper Chromatography and Analysis

This was described in sections 12(b), 12(c) and 12(d).

Results

1. Plasmid Analysis of 23 *Vibrio* species

As discussed earlier (pages 54-59) the virulence plasmid (pJm1, molecular weight 47mD), encoding an iron-uptake system, is an important virulence determinant of *V. anguillarum* strain 775. The incidence of plasmids in 23 strains of various *Vibrio* species, isolated from different geographical sources and from different species of fish or shellfish, was investigated.

Plasmids were isolated from the *Vibrio* strains using two different methods. In initial screening of *Vibrio* species for the presence of plasmids, the method of Kado and Liu (1981) was used and any plasmids detected were subsequently isolated by the method of Holmes and Quigley (1981).

1.1 Screening of Plasmid pJm1

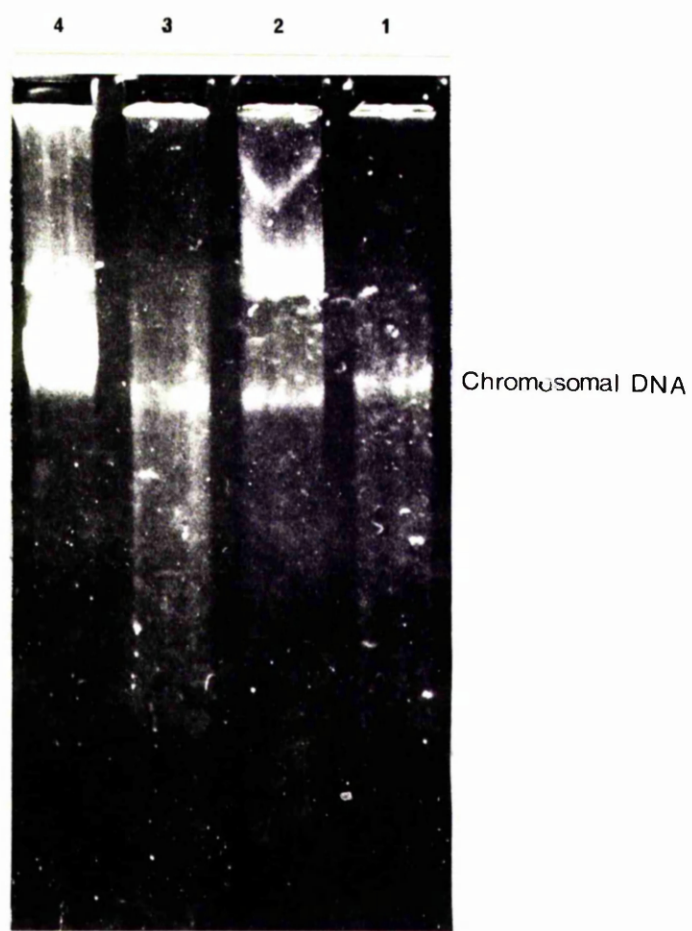
Since *V. anguillarum* 775 contains the plasmid pJm1, this strain was used to determine the optimum conditions for screening of the other *Vibrio* strains. *V. anguillarum* 775 was grown in shake flask cultures of 10ml NBS medium at 25°C and samples (1.5ml) were removed for plasmid analysis in the late exponential phase of growth (7h) and after overnight incubation. Plasmid preparations were obtained by the method of Kado and Liu (1981) and compared by electrophoresis (figure 10). Both samples contained contaminating chromosomal DNA, but this was consistently less in the 7h sample. Therefore, all subsequent samples were collected in late exponential phase of growth in NBS. *V. anguillarum* strain NCMB6 was used as a control strain lacking in plasmids.

1.2 Plasmid Screening of 23 *Vibrio* species

The 23 *Vibrio* species were analysed for plasmids as indicated above, and the molecular weights of any plasmids were roughly compared

Figure 10. Visualisation of plasmids from V. anguillarum strains 775 and NCMB6 in the late exponential phase of growth (7h) and after overnight incubation, in agarose gels

Lane 1	DNA from <u>V. anguillarum</u> strain NCMB6 after 7h incubation
Lane 2	DNA from <u>V. anguillarum</u> strain 775 after 7h incubation
Lane 3	DNA from <u>V. anguillarum</u> strain NCMB6 after overnight incubation
Lane 4	DNA from <u>V. anguillarum</u> strain 775 after overnight incubation



using pJM1 as a marker (figure 11 and table 8). Plasmids were detected in only 3 of the 23 strains, viz V. anguillarum strains 775 (pJM1), 4979 and 1197 with molecular weights of 45-50mD.

1.3 The Isolation of Plasmid DNA

The plasmids found in V. anguillarum strains 775, 1197 and 4979 were isolated by the method of Holmes and Quigley (1981) to remove contaminating chromosomal DNA. V. anguillarum NCMB6 was again used as a plasmid-less control strain. In this method, the length of time to which samples are heated to 100°C during the preparation is a critical parameter. Figure 12 shows that the plasmids were only detected after heating the samples for 40 seconds at 100°C; any deviation either by increasing or decreasing the time resulted in the loss of plasmid DNA.

2. Growth of 23 Vibrio species Under Conditions of Iron-Limitation

As discussed earlier (pages 54-59) the virulence plasmid pJM1 codes for an iron-uptake system in V. anguillarum strain 775 (Crosa, 1980) and loss of pJM1 plasmid gives a corresponding attenuation of virulence and loss of ability to grow under iron-limiting conditions. As the iron-uptake system is an important virulence determinant in the pathogenesis of V. anguillarum infections, the ability of 23 strains of Vibrio to grow under iron-limiting conditions was determined.

2.1 Determination of Free Iron in Culture Media

The amount of free ferric iron in all culture media used throughout this thesis was determined with the Sigma Iron Binding Capacity Kit. The amount of transferrin required to bind the available ferric iron was calculated and the chelation of iron was confirmed by subsequent determination of residual free iron. Table 9 shows the

Figure 11 Visualisation of plasmids from V. anguillarum
strains 775, 1197 and 4979 after 7h incubation
in agarose gels.

Lanes 1 and 2	DNA from <u>V. anguillarum</u> strain 775
Lane 3	DNA from <u>V. anguillarum</u> strain 1197
Lane 4	DNA from <u>V. anguillarum</u> strain 4979

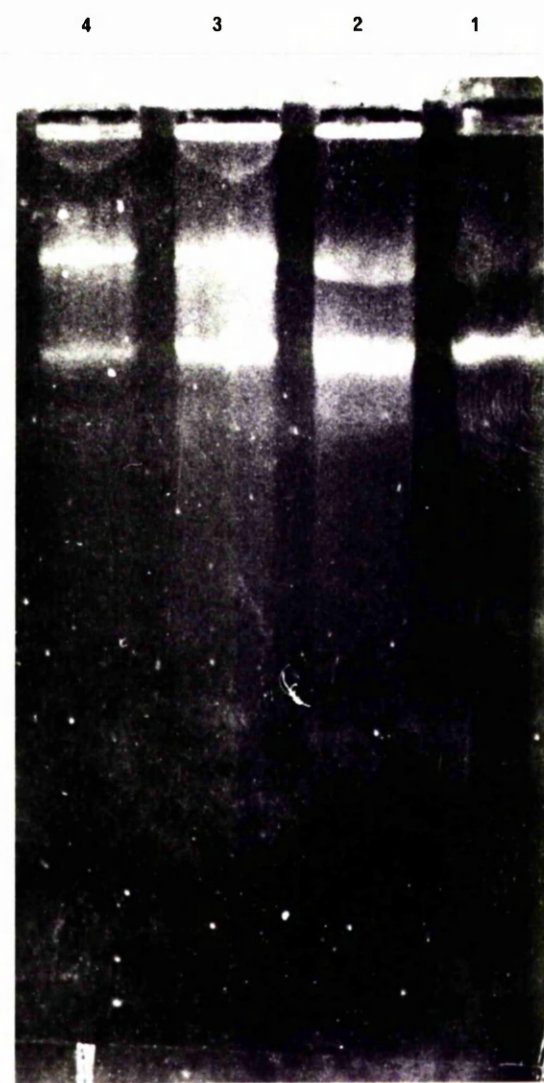


Table 8 The Molecular Weight of Plasmids found in
Three Strains of *Vibrio anguillarum*

<u>V. anguillarum</u>	Plasmid Content ^(a)
strain	(mD)
775 ^(b)	47
1197	45-50
4979	45-50

(a) Plasmids were detected by the method of Kado and Liu (1981)

(b) V. anguillarum strain 775 with a plasmid of known molecular weight was used as a standard marker.

Figure 12. Isolation of plasmids from V. anguillarum by the method of Holmes and Quigley (1981) and visualised in agarose gels

Lanes 1, 5 and 9. V. anguillarum strain 775 DNA heated for 30, 40 and 50 seconds of 100°C respectively

Lanes 2, 6 and 10. V. anguillarum strain 1197 DNA treated as above

Lanes 3, 7 and 11. V. anguillarum strain 4979 DNA treated as above

Lanes 4, 8 and 12. V. anguillarum strain NCMB6 DNA treated as above

12 11 10 9 8 7 6 5 4 3 2 1

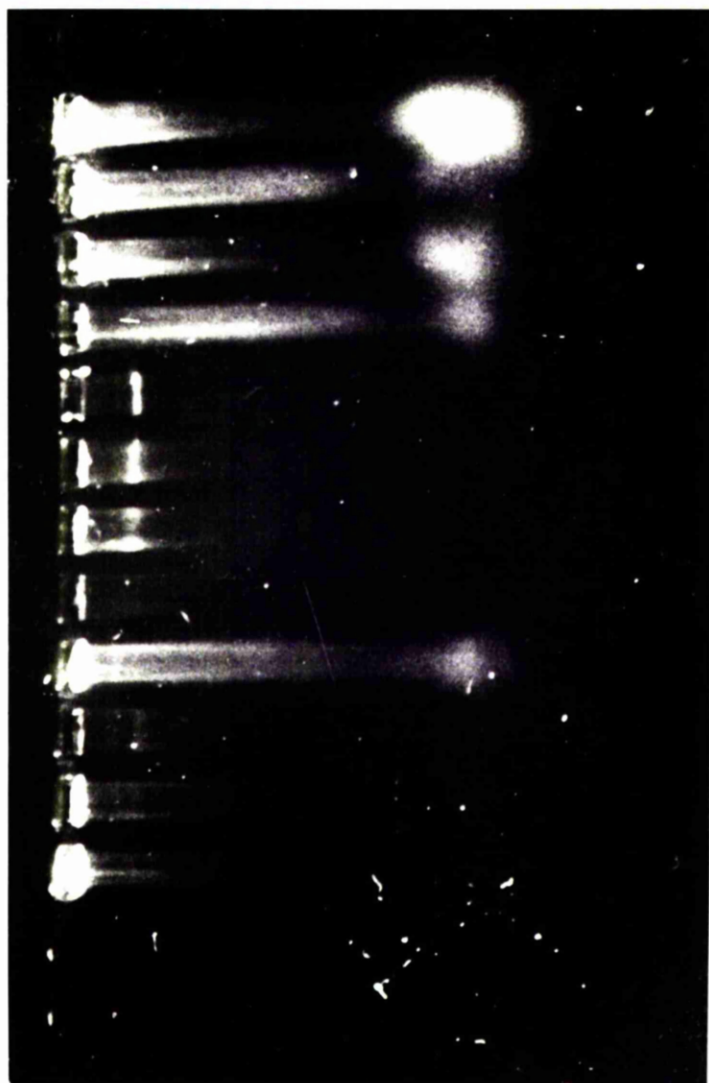


Table 9. Concentration of Ferric Iron in Culture Media and the
Concentration of Transferrin Added to Chelate Available Iron

Culture medium	Ferric Iron ^(a) concentrations (μ M)	Final Concentration of ^(b) Transferrin (μ g/ml)
Oxoid Nutrient Broth		
No. 2 plus 1.5% NaCl	8.86	500
Vibrio Minimal Medium	1-1.5	70
Tris Succinate Medium	1.5-2.0	100

(a) Determined using "The Iron Binding Capacity Kit" (Sigma).

(b) An excess of 10% was added.

amount of free ferric iron typically found in each culture medium and the amount of transferrin to ensure an iron-limiting medium.

2.2 Growth of 23 *Vibrio* strains Under Conditions of Iron Limitation

To determine the ability of the *Vibrio* strains to grow under iron-limiting conditions, each strain was grown in 50ml shake flask cultures of three different media. Nutrient broth plus salt (NBS) was used to determine a normal, reference, growth pattern and NBS plus transferrin (NBST) was used to determine the growth pattern in an iron-limiting medium. If growth was inhibited in NBST, the addition of free ferric iron (NBST plus FeCl_3) would overcome the inhibition if it was due to the depletion of available iron in the medium NBST.

When growth of the strains was compared in the three media (figure 13), the 23 strains fell into one of three patterns shown in figure 13. Only 3 strains, *V. tubiashi* strain 1337 and *Vibrio* species 1338 and B51 were unable to grow under iron-limiting conditions. *V. anguillarum* strain 2981 and *V. tubiashi* strain 1336 had a longer lag phase when grown in NBST, but growth of all other strains was not affected by iron-limitation. Therefore, an iron-uptake system appeared to be present in 20 of the 23 *Vibrio* strains and there was no apparent correlation between the presence of a plasmid (40-50mD) and ability to grow under iron-limiting conditions (Table 10).

3. Cell Envelope Proteins Induced Under Iron Limitation

Since 20 of the 23 *Vibrio* strains grew under iron-limiting conditions, the iron-uptake systems of 10 of the *V. anguillarum* strains were investigated in more detail by preparing cell envelope fractions from cells grown in the presence and absence of transferrin.

Figure 13

Growth Patterns of Vibrio anguillarum.

Three different patterns of growth were noted,
represented here as Pattern A (strain 775),
Pattern B (strain 2981) and Pattern C (strain 1338).

○—○ Growth in NBS

▲—▲ Growth in NBST

▴—▴ Growth in NBST plus FeCl_3 .

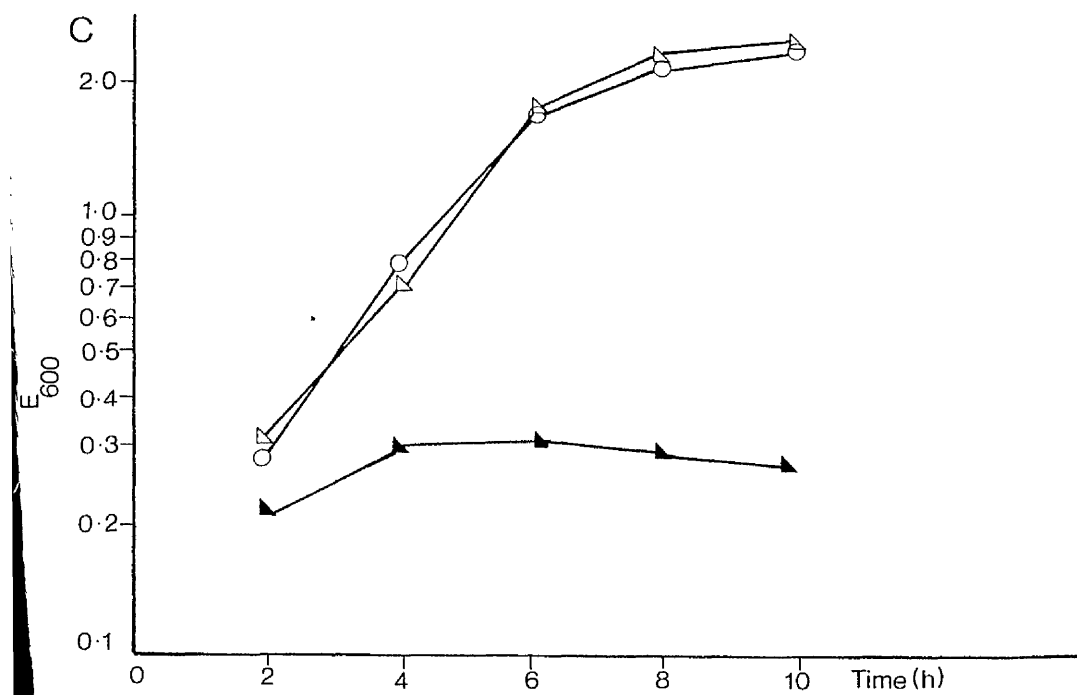
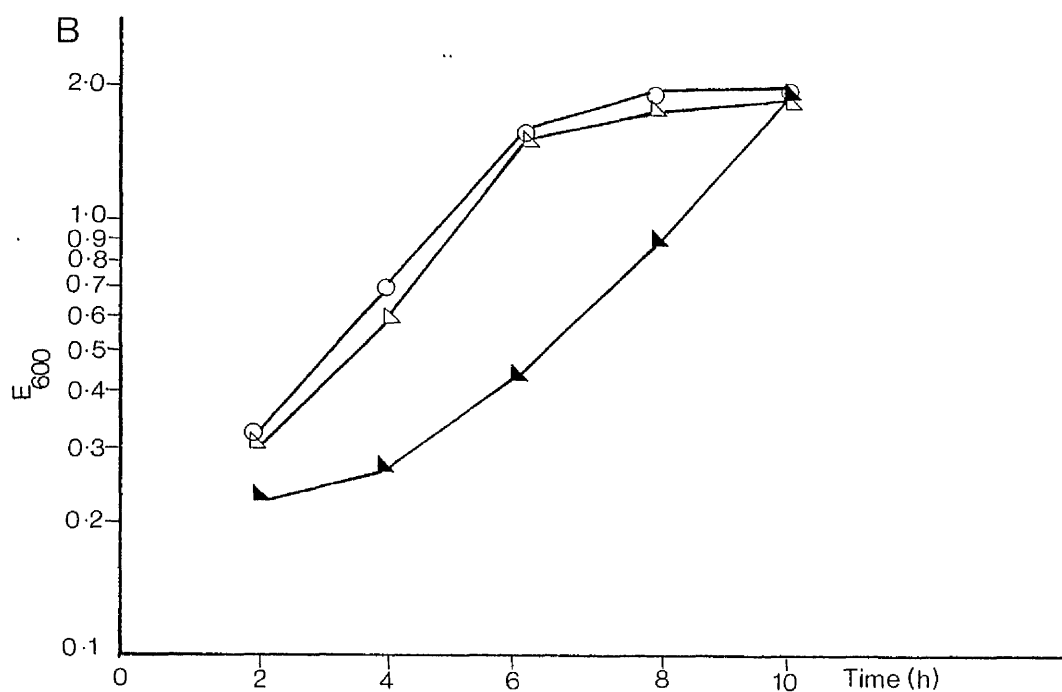
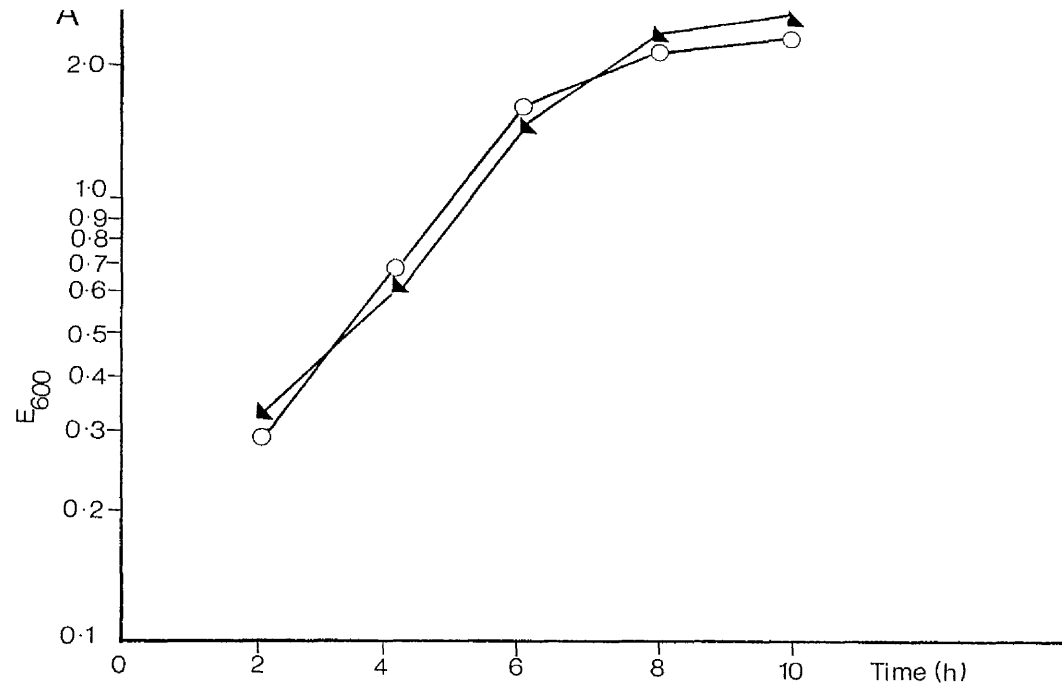


Table 10. Plasmid Content and Growth Under Iron-Limiting Conditions
for 23 Vibrio strains

<u>Vibrio</u> strain	Plasmid content (mD)	Growth Under ^(a) Iron-Limitation	Growth Pattern (b)
<u>V. anguillarum</u> 775	+ 47	++	A
1197	+ 45-50	++	A
4979	+ 45-50	++	A
NCMB6	-	++	A
636	-	++	A
827	-	++	A
1445	-	++	A
91079	-	++	A
5679	-	++	A
2981	-	++	B
2164	-	++	A
2165	-	++	A
2166	-	++	A
<u>V. tubiashi</u> 1336	-	++	B
1337	-	-	C
1340	-	++	A
<u>V. alginolyticus</u>			
1339	-	++	A
<u>Vibrio</u> species			
1338	-	-	C
B51	-	-	C
B55	-	++	A
B2	-	++	A
T61	-	++	A
B1	-	++	A

(a) ++ Growth in iron-limiting media after 8h.

- No growth in iron-limiting media after 8h.

(b) For Growth Patterns A, B and C, see figure 13.

Optimum conditions to observe new envelope proteins produced by the V. anguillarum strains when grown under iron-limiting conditions were determined using V. anguillarum strains 775 and NCMB6 and two different preparation techniques; the Chemical Lysis and X-Press method (Yamato et al., 1975; Owen et al., 1982) and the EDTA-Extraction Procedure (Zollinger et al., 1972). Strain 775 was chosen as it is known to produce two new outer membrane proteins under iron-limiting conditions and strain NCMB6, one new envelope protein (Crosa & Hodges, 1981; Crosa, 1981).

3.1. Detection of New Cell Envelope Proteins

Cell envelope preparations of V. anguillarum strains 775 and NCMB6, grown in shake flask cultures of NBS or NBST were made by both the procedures described above (see pages 77-78) and compared in 12.5% polyacrylamide gels

With the chemical lysis and X-press method of Yamato et al. (1975) and Owen et al. (1982) no new envelope proteins were detected when either strain was grown in the presence or absence of transferrin. However using the EDTA extraction procedure of Zollinger et al. (1972) faint new protein bands were detected when both strains were grown under iron-limiting conditions. V. anguillarum 775 produced two new proteins and V. anguillarum NCMB6 produced one new protein (figure 14), confirming previous investigations.

The molecular weights of these proteins were estimated as 72.5kD and 79kD for strain 775 and 69kD for NCMB6.

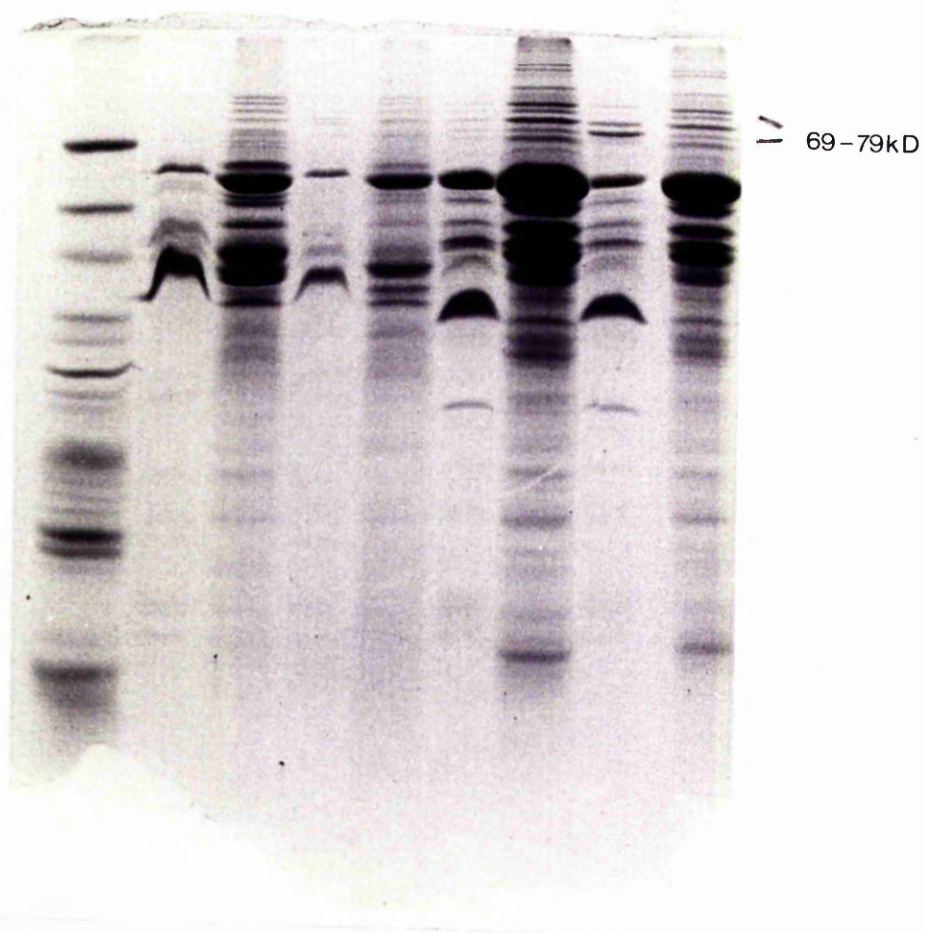
Since this procedure resulted in detection of new envelope proteins, it was decided to discontinue the use of the chemical lysis and X-press procedure.

Figure 14. SDS-PAGE of cell envelope proteins from V. anguillarum strains 775 and NCMB6 detected by the Chemical lysis and X-Press method and the EDTA extraction procedure.

Cell envelope fractions from V. anguillarum strains 775 and NCMB6 grown in iron-limiting and iron-replete media were prepared by the Chemical lysis and X-Press method (Yamato et al., 1975; Owen et al., 1982) and the EDTA extraction procedure (Zollinger et al., 1972) and compared by SDS-PAGE. Protein bands were visualised by Coomassie Blue staining.

Lane 1, V. anguillarum strain 775 (cultured in NBS), EDTA extraction procedure; lane 2, V. anguillarum strain 775 (cultured in NBS), chemical lysis and X-Press method; lane 3, V. anguillarum strain 775 (cultured in NBST), EDTA extraction procedure; lane 4, V. anguillarum strain 775 (cultured in NBST), Chemical lysis and X-Press method; lane 5, V. anguillarum strain NCMB6 (cultured in NBS), EDTA extraction procedure; lane 6, V. anguillarum strain NCMB6 (cultured in NBS), Chemical lysis and X-Press method; lane 7, V. anguillarum strain NCMB6 (cultured in NBST), EDTA extraction procedure; lane 8, V. anguillarum strain NCMB6 (cultured in NBST), Chemical lysis and X-Press method.

standard 1 2 3 4 5 6 7 8



3.2 Improvements in the EDTA Extraction Procedure

Since the new envelope proteins were only faintly visible on polyacrylamide gels a number of possible improvements to the EDTA extraction procedure were investigated.

As explained more fully elsewhere (pages 78-79) in samples subjected to centrifugation at 84,000g for 2h at 4°C, followed by standing overnight at 4°C, a small yellow opalescent pellet settled at the bottom of the centrifugation tube. This is represented diagrammatically in figure 15. The material was collected and analysed by SDS-PAGE (figure 16).

The new envelope proteins produced under iron-limiting conditions with V. anguillarum 775 and NCMB6 were more pronounced in the yellow fraction as the background material was considerably reduced. This separation of material after centrifugation was a useful step in purification as shown by the protein content of the envelope samples before and after the improvement step (Table 11). The opalescent pellet contained approximately one fifth of the protein content of the original pellet which presumably contained a high amount of contaminating material.

3.3 Preparation of Immunoglobulin Fractions of Antisera to Cell Envelope Proteins of V. anguillarum 775 and NCMB6.

For two-dimensional electrophoresis and immunoelectroblotting analysis, immunoglobulins to cell envelope proteins, in particular proteins associated with iron-limitation, were prepared. To obtain strong polyvalent antisera against both V. anguillarum strains 775 and NCMB6, two New Zealand white rabbits per strain were injected intramuscularly with cell envelope preparations emulsified in complete Freund's adjuvant. The cell envelopes were prepared by the EDTA extraction

Figure 15. Preparation of cell envelopes from the Improved EDTA
Extraction procedure.

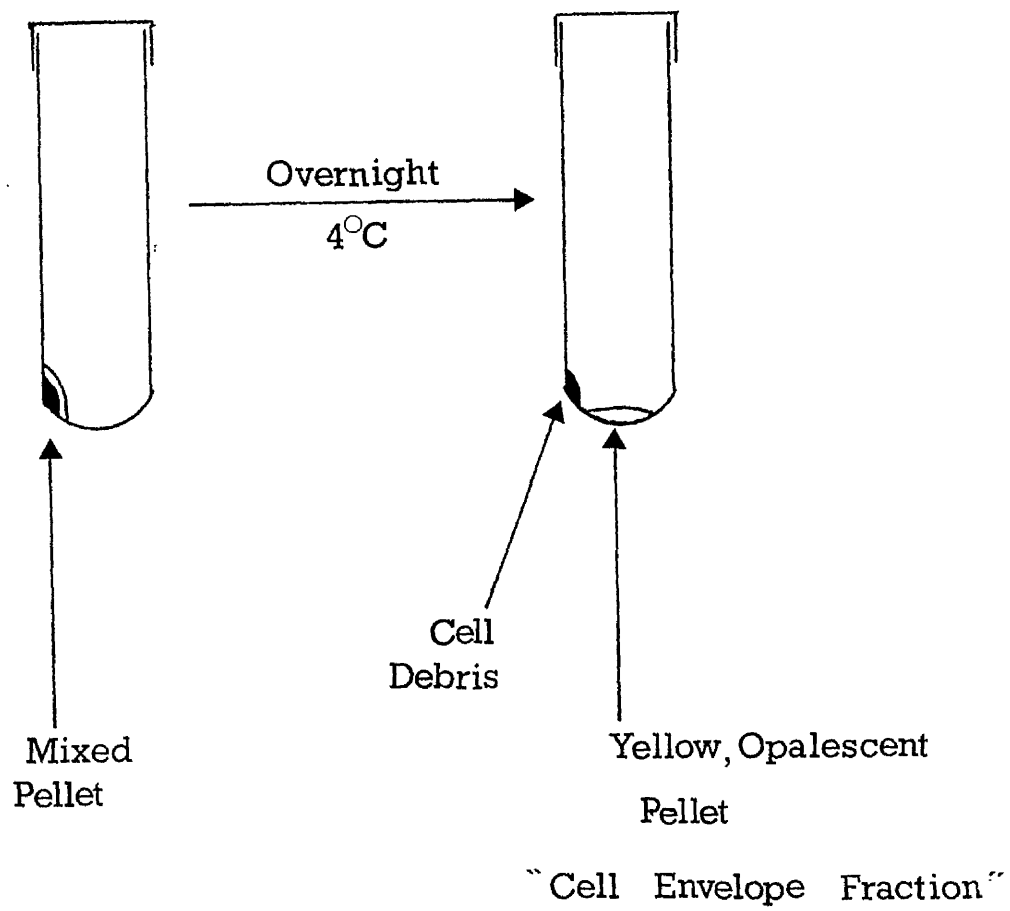


Figure 16. SDS-PAGE of cell envelope proteins associated with iron-limitation in V. anguillarum strains 775 and NCMB6 prepared by the improved EDTA extraction procedure.

Cell envelope fractions from V. anguillarum strains 775 and NCMB6 grown in iron-limiting and iron-replete media were prepared by the improved EDTA extraction procedure and compared by SDS-PAGE. Protein bands were visualised by Coomassie Blue staining.

figure (a) Lanes 1 and 2, V. anguillarum strain 775 cultured in NBS;
lanes 3 and 4, V. anguillarum strain 775 cultured in NBST.
figure (b) Lanes 1 and 2, V. anguillarum strain NCMB6 cultured in NBS;
lanes 3 and 4, V. anguillarum strain NCMB6 cultured in NBST.

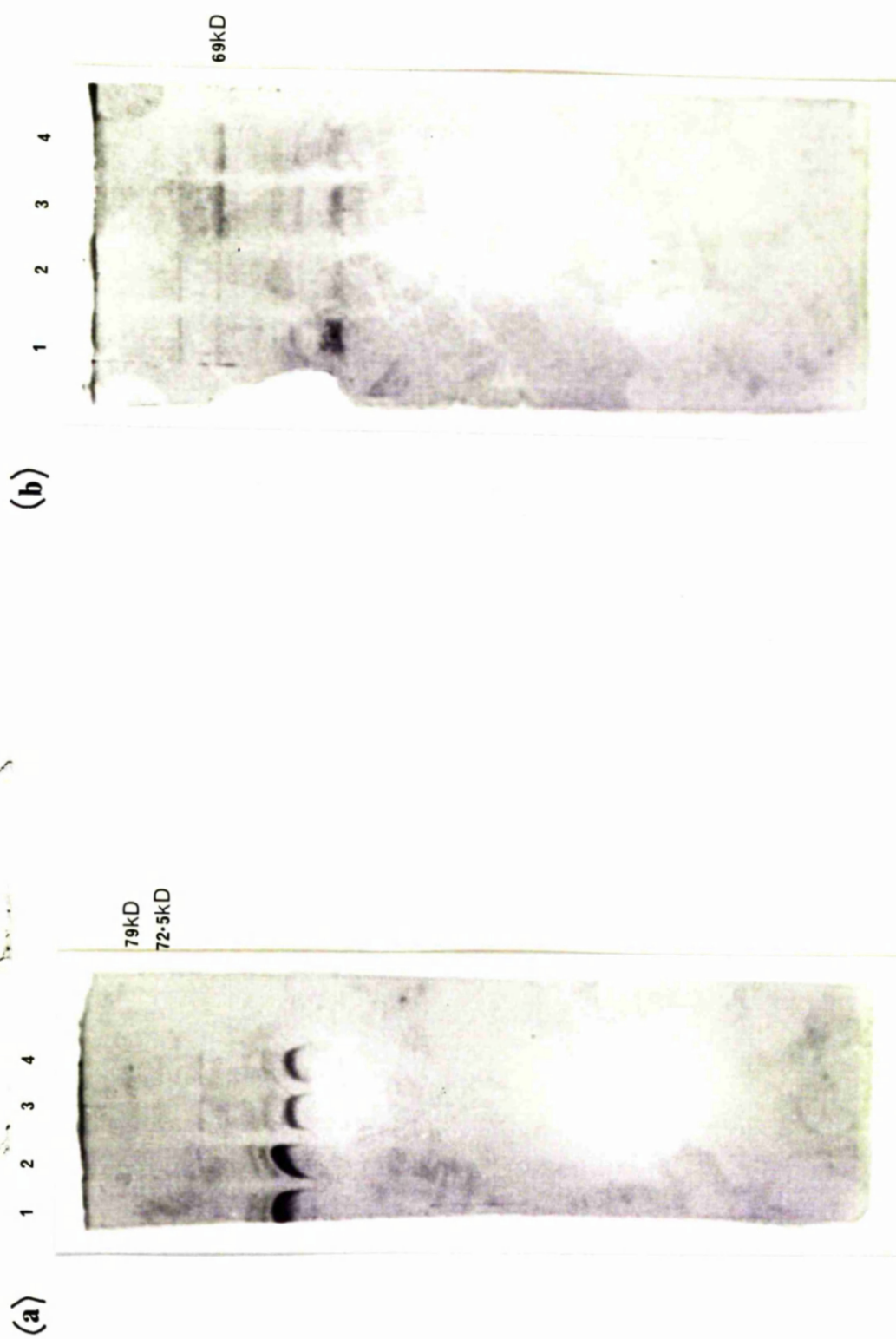


Table 11. Protein Concentrations in Three Cell Envelope Preparations
of *V. anguillarum* strains 775 and NCMB6

Cell Envelope Preparation	Protein concentration (mg./ml)	
	775	NCMB6
Chemical Lysis and		
X-Press Method	9	7.5
EDTA Extraction procedure	1	0.5
Improved EDTA extraction procedure	0.25	0.1

procedure of Zollinger et al. (1972) from bacterial cells grown in NBST medium and therefore the antiserum would contain a wide range of immunoglobulins to both proteins and LPS. The antisera were pooled and antigen-antibody agglutination titre tests and gel diffusion were performed. This first collection of antisera will be termed crude antiserum of either V. anguillarum 775 or NCMB6.

After the first test bleeds rabbits were given intravenous booster injections of cell envelope fractions prepared by the improved EDTA extraction procedure. In this way it was hoped that one could enhance the production of immunoglobulins to the envelope proteins associated with iron limitation. Further test bleeds were taken and booster injections were carried out every 2-3 months to maintain the immunoglobulin titres against envelope proteins of V. anguillarum 775 and NCMB6.

Appropriate test bleeds were pooled and 5ml amounts of antiserum were absorbed with cell envelope fractions (prepared by the EDTA extraction procedure) from bacterial cells grown in NBS medium (as outlined in Materials and Methods) until there was no residual reaction in gel diffusion or by antibody-antigen agglutination (Figures 17 and 18).

The antisera for both V. anguillarum 775 and NCMB6 are referred to as follows:

1. Crude antiserum - Pooled from the initial test bleeds.
2. Unabsorbed antiserum - Pooled from test bleeds collected after intravenous booster injections.
3. Absorption antiserum A - Pooled antiserum after first stage absorption (Figures 17(b) and 18(b)).
4. Absorption antiserum B - Pooled antiserum after second stage absorption (Figures 17(c) and 18(c)).

Figure 17. Reactions in gel diffusion of Antiserum to a cell envelope fraction of V. anguillarum strain 775 grown under iron-limitation and absorbed with cell envelope fractions of V. anguillarum strain 775 grown in iron-replete medium.

The reaction in gel diffusion of unabsorbed and absorbed antisera was visualised with cell envelope fractions from V. anguillarum strain 775 cells grown in NBS medium (column I) and cells grown in NBST medium (column II).

- (a) Unabsorbed Antiserum
- (b) Absorption Antiserum A
- (c) Absorption Antiserum B.

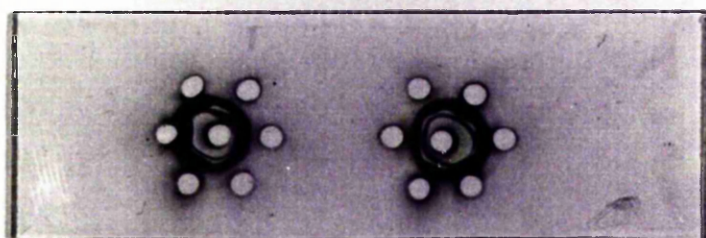
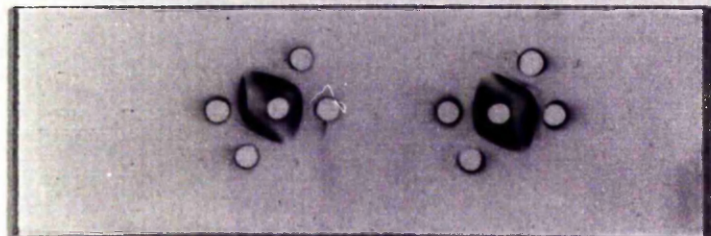
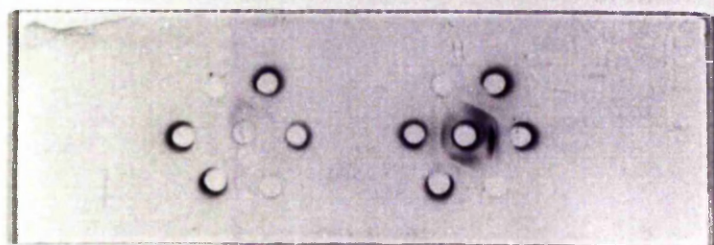
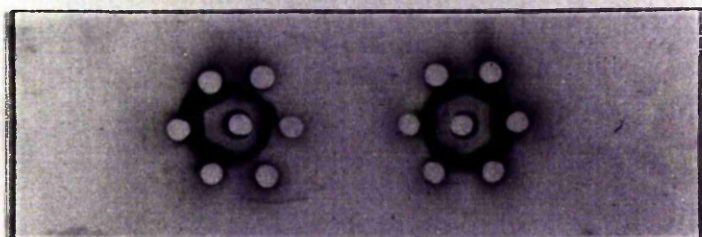
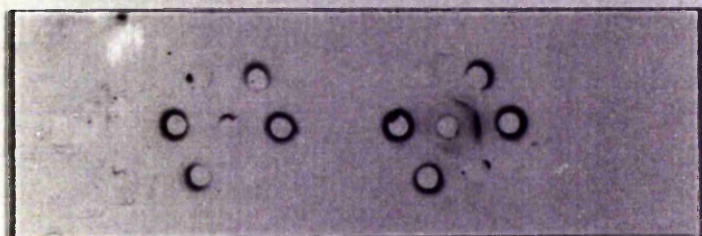
I**II****a****b****c**

Figure 18. Reactions in gel diffusion of Antiserum to a cell envelope fraction of V. anguillarum strain NCMB6 grown under iron-limitation and absorbed with cell envelope fractions of V. anguillarum strain NCMB6 grown in iron-replete medium.

The reaction in gel diffusion of unabsorbed and absorbed antisera was visualised with cell envelope fractions from V. anguillarum strain NCMB6 cells grown in NBS medium (column I) and cells grown in NBST medium (column II).

- (a) Unabsorbed Antiserum
- (b) Absorbed Antiserum A
- (c) Absorbed Antiserum B.

I**II****a****b****c****NO REACTION**

Crude immunoglobulin fractions of antisera were purified by precipitation with $(\text{NH}_4)_2\text{SO}_4$ and were stored at 4°C or -20°C .

3.4 Immunological Analysis of Cell Envelopes Prepared from *V. anguillarum* strains 775 and NCMB6 by Electroblotting

Cell envelope proteins of *V. anguillarum* 775 and NCMB6 which were associated with iron-limitation were initially demonstrated immunologically by separation on 12.5% polyacrylamide gels, electrotransfer to nitrocellulose paper and reaction with antisera described above (section 3.3). Antibody-antigen reactions on the nitrocellulose paper were visualised using sheep anti-rabbit-horseradish peroxidase conjugate (HRP) with 4-chloro-naphthol as substrate (Figures 19 and 20).

Using *V. anguillarum* 775 absorbed antiserum B, 4 protein bands were detected from envelope fractions of bacterial cells grown in NBST medium, but only 2 protein bands appeared from envelope preparations from bacteria grown in NBS medium (Figure 19). The two extra protein bands corresponded to the proteins of 72.5kD and 79kD detected on a parallel gel. This suggests that these proteins are associated with the iron-uptake system(s) of *V. anguillarum* 775.

These proteins could not be detected using unabsorbed antiserum or absorbed antiserum A due to interference by immunoglobulins to LPS.

Using absorbed antiserum A to *V. anguillarum* NCMB6 a range of protein bands were visualised on the nitrocellulose paper for both envelope preparations of bacterial cells grown in NBS or NBST media (Figure 20). A more intense antibody-antigen reaction was visible for a protein, corresponding to molecular weight 69kD on a parallel gel, in the envelope fraction of bacterial cells grown under iron-limiting conditions. Although a small amount of the 69kD protein was present in the cell envelope when iron was freely available, it was produced in

Figure 19. Immunodetection of proteins associated with iron-limitation in the cell envelope of V. anguillarum strain 775 by electroblotting.

Nitrocellulose blot analysis of cell envelope proteins from V. anguillarum strain 775 grown under iron-limiting and iron-replete conditions separated by SDS-PAGE and reacted with Absorbed Antiserum B against V. anguillarum strain 775.

Lanes 1 and 2. A cell envelope fraction from V. anguillarum strain 775 cells grown in NBST medium.

Lanes 3 and 4. A cell envelope fraction from V. anguillarum strain 775 cells grown in NBS medium.

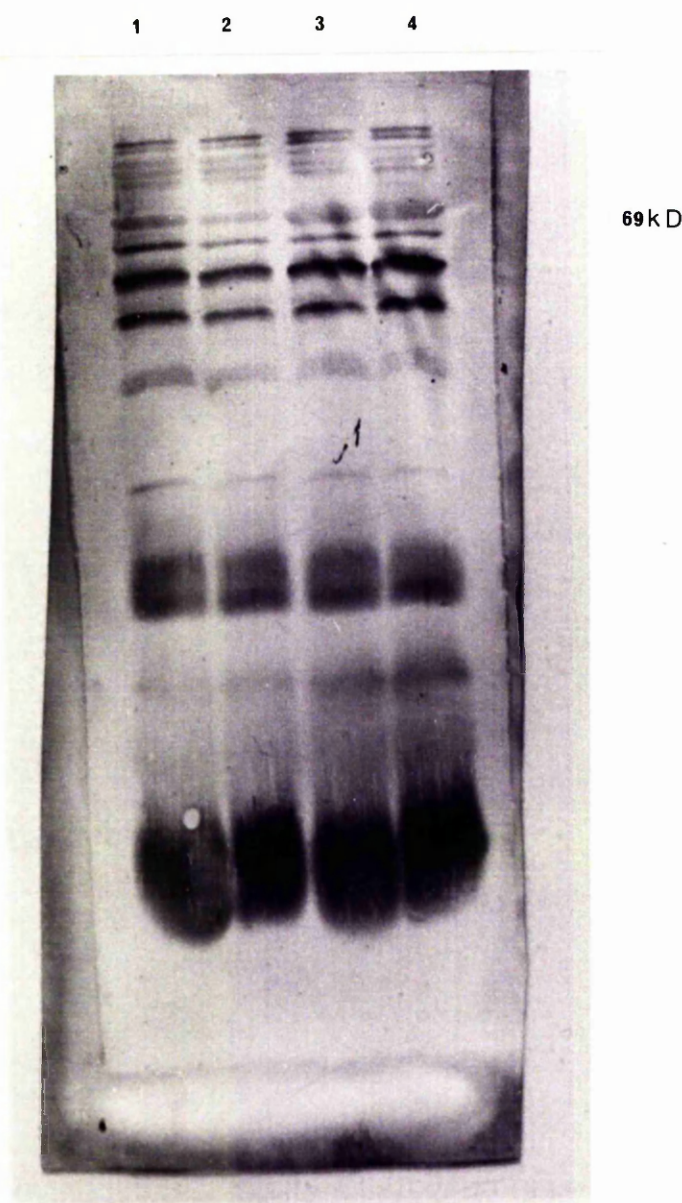


Figure 20. Immunodetection of proteins associated with iron-limitation in the cell envelope of V. anguillarum strain NCMB6 by electroblotting.

Nitrocellulose blot analysis of cell envelope proteins from V. anguillarum strain NCMB6 grown under iron-limiting and iron-replete conditions separated by SDS-PAGE and reacted with Absorbed Antiserum A against V. anguillarum strain NCMB6.

Lanes 1 and 2. A cell envelope fraction from V. anguillarum strain NCMB6 grown in NBS medium.

Lanes 3 and 4. A cell envelope fraction from V. anguillarum strain NCMB6 grown in NBST medium.



larger amounts under conditions of iron-limitation and therefore appears to be associated with the iron-uptake system(s) of V. anguillarum NCMB6.

3.5 Two Dimensional Electrophoresis of Cell Envelopes Prepared from V. anguillarum 775 and NCMB6

Cell envelope proteins associated with iron-limitation were also detected immunologically by two-dimensional immunoelectrophoresis. Envelope preparations (using the Improved EDTA extraction procedure) from bacterial cells grown in NBS and NBST media were compared using absorbed antiserum A to V. anguillarum strains 775 and NCMB6 previously described in section 3.3.

After electrophoresis in the second direction the antibody-antigen precipitin arcs were visualised by staining with a saturated solution of nigrosine black.

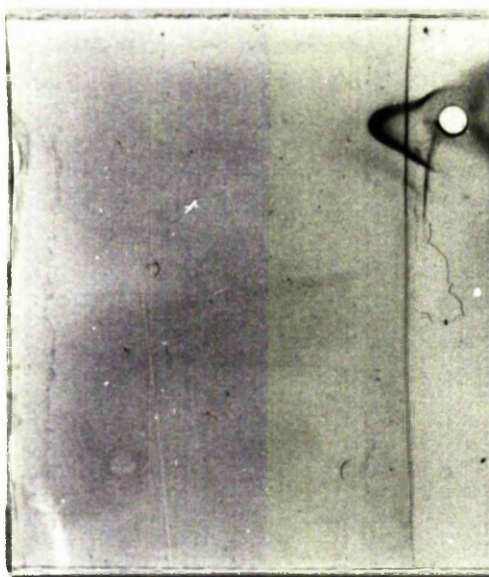
With both V. anguillarum 775 and NCMB6 one extra precipitin arc could be seen with envelope fractions prepared from bacterial cells grown under iron-limiting conditions (Figures 21 and 22) and these could be designated as reference precipitin arcs for cell envelope proteins associated with iron-limitation. However, this procedure required a relatively large amount of antiserum and was not as sensitive as electro-transfer blotting in demonstrating proteins involved in iron-uptake (with both strains, absorbed antiserum B gave no precipitin arcs to cell envelope fractions of V. anguillarum strains 775 or NCMB6).

Therefore, for further experiments to detect antigenic cross-reaction of envelope proteins associated with iron-limitation in 10 different strains of V. anguillarum, electroblotting onto nitrocellulose paper and subsequent antigen-antibody reactions was used with absorbed

Figure 21. Immunodetection of proteins associated with iron-limitation in the cell-envelope of V. anguillarum strain 775 by two-dimensional electrophoresis.

Two-dimensional immunoelectrophoresis of cell envelope proteins from V. anguillarum strain 775 grown under iron-limiting (figure A) and iron-replete (figure B) conditions with Absorbed Antiserum A to V. anguillarum strain 775. The position of the extra precipitin arc seen with the cell envelope fraction prepared from cells grown under iron-limitation is shown by an arrow on figure A.

B



A

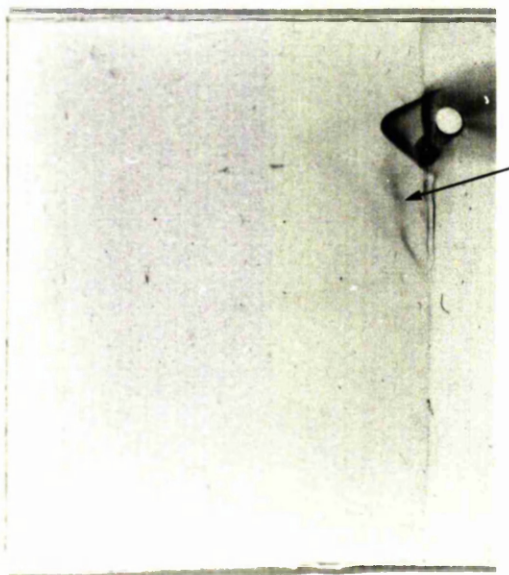
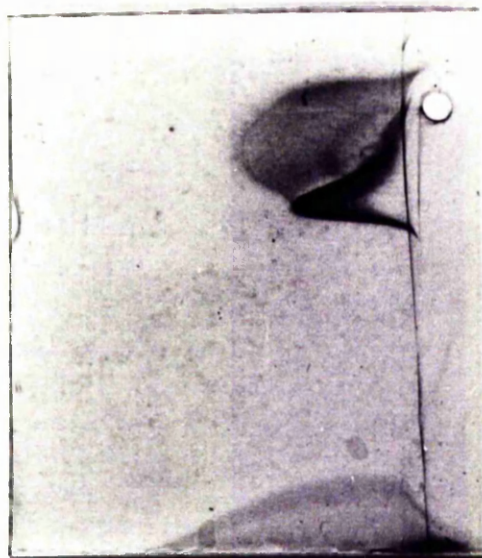


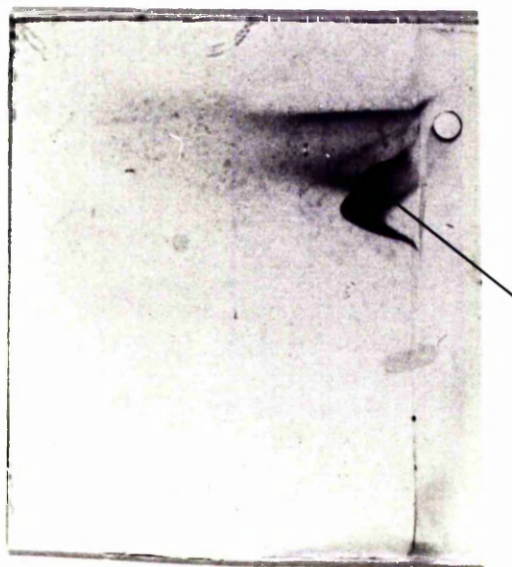
Figure 22. Immunodetection of proteins associated with iron-limitation in the cell envelope of V. anguillarum strain NCMB6 by two-dimensional electrophoresis.

Two-dimensional immunoelectrophoresis of cell-envelope proteins from V. anguillarum strain NCMB6 grown under iron-limiting (figure A) and iron-replete (figure B) conditions with Absorbed Antiserum A to V. anguillarum strain NCMB6. The position of the extra precipitin arc seen with the cell envelope fraction prepared from cells grown under iron-limitation is shown by an arrow on figure A.

B



A



antiserum B to V. anguillarum 775 as this appeared to be the most specific antiserum for envelope proteins associated with iron-limitation.

4. Cell Envelope Proteins Associated with Iron-Limitation in Ten Strains of V. anguillarum

To compare the cell envelope proteins associated with iron-limitation in ten strains of V. anguillarum several techniques were employed using cell envelope preparations, made by the Improved EDTA extraction procedure, of the 10 strains grown in NBS or NBST media. Previous investigations (Toranzo et al., 1985) showed that the 86kD protein, associated with an iron-uptake system, coded on the plasmid pJM1 and similar plasmid-containing strains of V. anguillarum isolated in Spain, cross-reacted with monoclonal antiserum raised against the 86kD protein, OM2. Antigenic similarities among the proteins produced by 10 V. anguillarum strains under iron-limiting conditions were investigated.

4.1 Determination of Cell Envelope Proteins Associated with Iron-Limitation

As V. anguillarum strains 775 and NCMB6 produced two and one new envelope protein(s) respectively under iron-limiting conditions, the cell envelope profiles of 8 other V. anguillarum strains were compared by 12.5% polyacrylamide gels. One to three new envelope proteins appeared when the bacterial cells were grown under iron-limiting conditions (Figure 23). The molecular weights of these proteins ranged from 68kD to 79kD (Table 12).

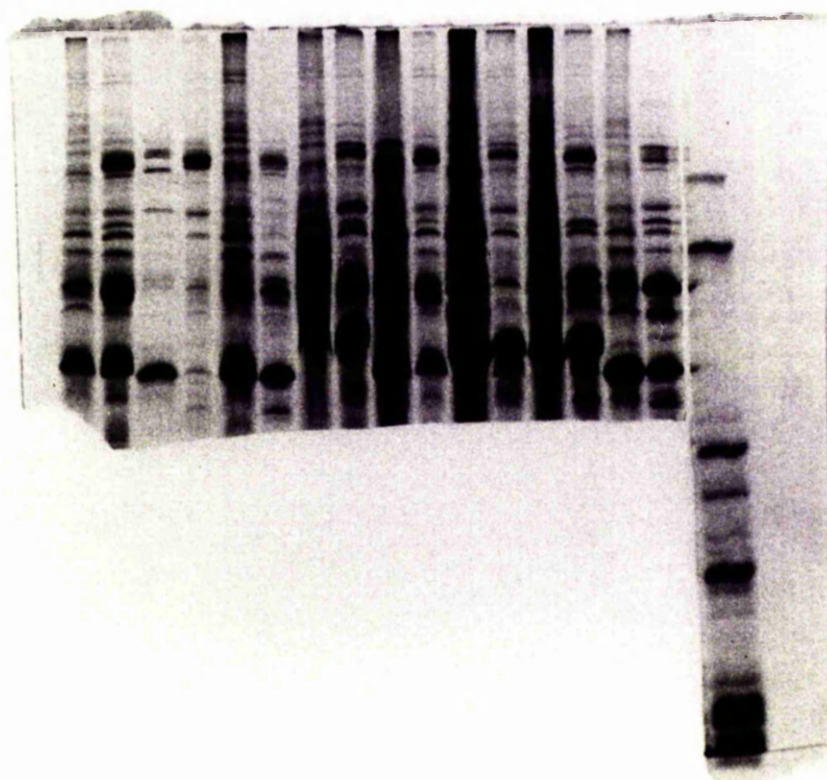
4.2 Immunological Comparison of the Cell Envelope Proteins Associated with Iron-Limitation

Figure 23. SDS-PAGE of cell envelope proteins associated with iron-limitation in 8 V. anguillarum strains.

Each strain was grown in iron-replete medium (left lane of each pair of lanes) or in iron-limiting medium (right lane of each pair of lanes) and cell envelope fractions were prepared by the improved EDTA extraction procedure. The fractions were compared on SDS-PAGE and protein bands visualised with coomassie blue staining.

Lanes 1 and 2, V. anguillarum strain 636; lanes 3 and 4, V. anguillarum strain 827; lanes 5 and 6, V. anguillarum strain 1445; lanes 7 and 8, V. anguillarum strain 1197; lanes 9 and 10, V. anguillarum strain 91079; lanes 11 and 12, V. anguillarum strain 2981; lanes 13 and 14, V. anguillarum strain 4979; lanes 15 and 16, V. anguillarum strain 5679.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 standard



69-79kD

Table 12. Estimated Molecular Weights of Proteins Associated with
Iron Limitation in 10 *V. anguillarum* Strains

<u><i>V. anguillarum</i></u>	No. of Proteins	Molecular Weight
Strain	Associated with	(kD)
	Iron-Limitation	
NCMB6	1	69
636	1	71
827	1	71
91079	1	72
2981	1	73
4979	1	73
775	2	72.5 ; 79
1197	2	73 ; 78
5679	2	73 ; 78
1445	3	68 ; 72 ; 73

Estimated from an SDS-PAGE gel in 12.5% polyacrylamide.

Envelope protein preparations, from bacterial cells of ten V. anguillarum strains grown in NBST medium, were separated by SDS-PAGE, electrotransferred to nitrocellulose paper and reacted with absorbed antiserum B to V. anguillarum strain 775. After detection of antibody-antigen reactions with substrate, two major bands were noted in all samples (Figure 24) and these proteins appear to be the major antigenic proteins in the cell envelope of V. anguillarum. The protein bands associated with iron-limitation varied with different strains. Two protein bands were detected in cell envelope samples of V. anguillarum strains 775, 1197 and 5679; all other strains showed only one protein band.

Therefore, one antigenically related envelope protein associated with iron-limitation was detected in all ten V. anguillarum strains and in two of the strains, a second protein cross-reacted with immunoglobulins to another V. anguillarum 775 envelope protein apparently involved in iron-uptake.

4.3 Iron-Binding Proteins detected by Ferene S Staining

Recently, Ching-Ming (1985) reported a specific stain for iron-binding proteins. When this method was used with human transferrin (70 µg/ml, as a positive control) and cell envelope preparations of V. anguillarum grown in an iron-limited medium, only transferrin reacted positively to the Ferene S stain (Figure 25(a)).

However, when the staining procedure was modified (see Materials and Methods) and an additional step was introduced in which the slab gel was immersed in a 1% (w/v) solution of FeCl_3 before staining with Ferene S stain, a more intense band was seen with transferrin and a range of protein bands were stained in lanes corresponding to cell envelopes of eight V. anguillarum strains (Figure 25(b)).

Figure 24. Immunodetection of proteins associated with iron-limitation in the cell envelope of V. anguillarum strains by electroblotting.

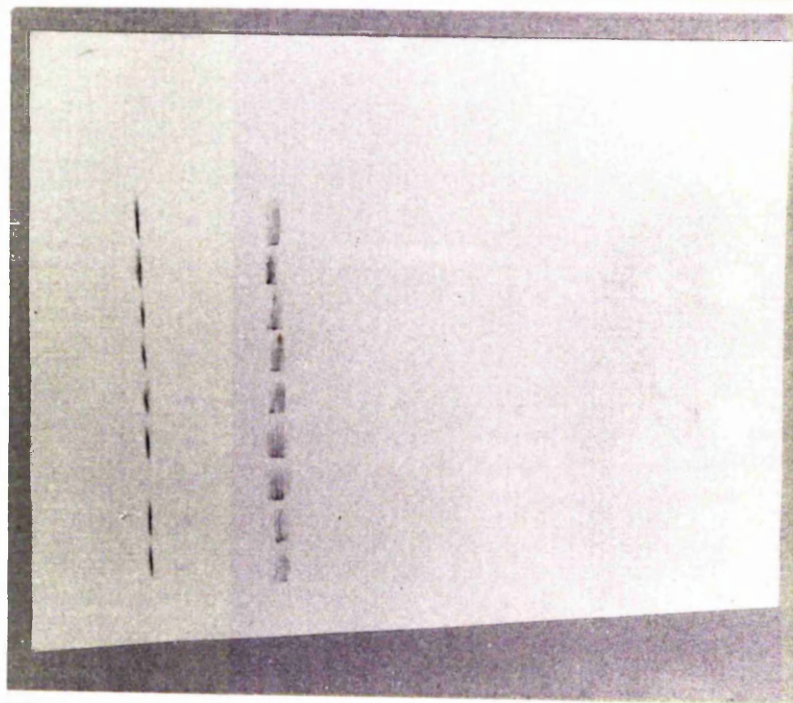
Nitrocellulose blot analysis of cell envelope proteins from V. anguillarum strains grown under iron-replete conditions (figure a) and iron-limiting conditions (figure b), separated by SDS-PAGE, and reacted with Absorbed Antiserum B against V. anguillarum strain 775.

figure a. lane 1, V. anguillarum strain NCMB6; lane 2, V. anguillarum strain 636; lane 3, V. anguillarum strain 1197; lane 4, V. anguillarum strain 5679; lane 5, strain 4979; lane 6, V. anguillarum strain 91079; lane 7, V. anguillarum strain 827; lane 8, V. anguillarum strain 1445; lane 9, V. anguillarum strain 2981.

figure b. lanes 1-9 as above.

1 2 3 4 5 6 7 8 9

(a)



(b)

-- 69-79kD --

1 2 3 4 5 6 7 8 9

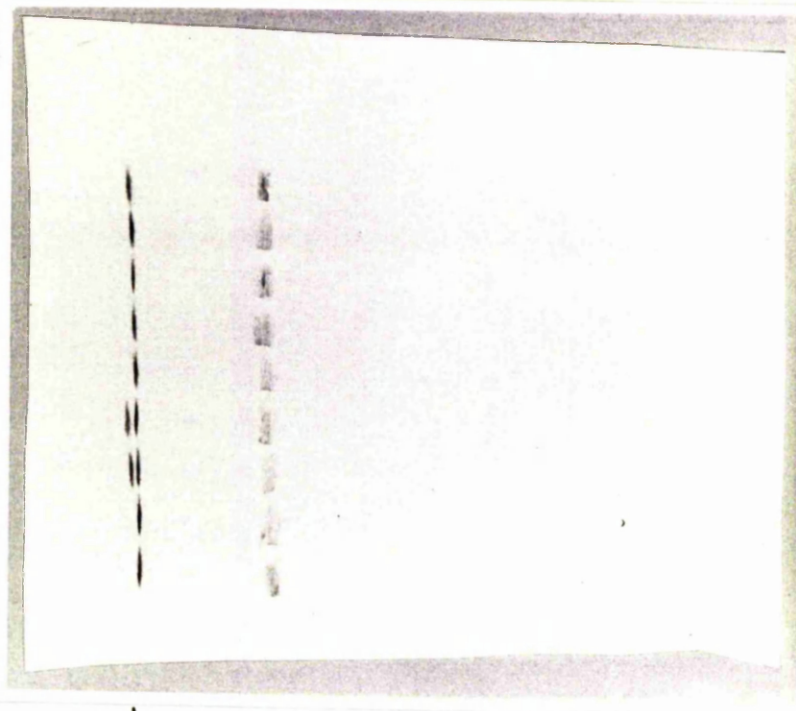


Figure 25. SDS-PAGE of iron-binding proteins detected by
Ferene S staining.

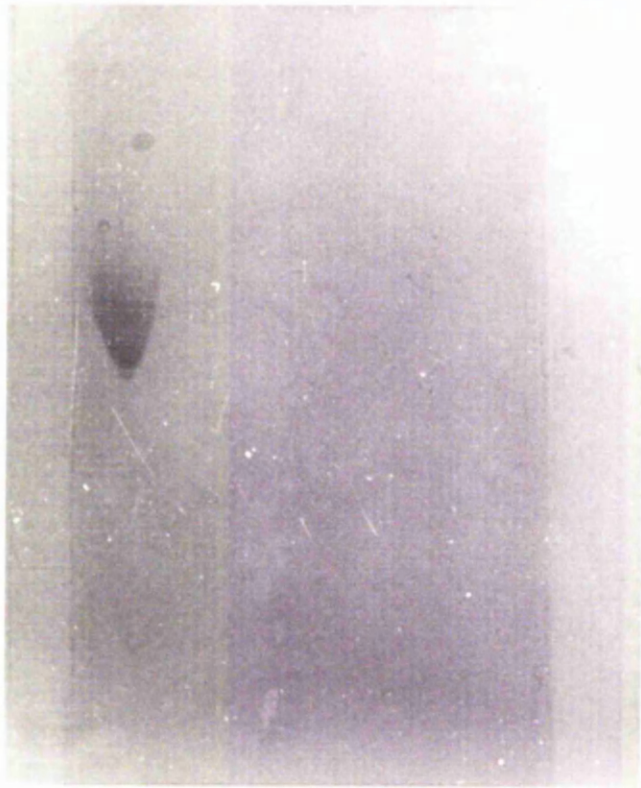
Human transferrin (70 $\mu\text{g./ml}$) and cell envelope fractions from eight V. anguillarum strains grown in NBST medium were separated on SDS-PAGE and iron-binding proteins detected with Ferene S stain. Figures (a) and (b) show the range of protein bands visualised when an additional step, in which the gel was immersed in 1% (w/v) solution of FeCl_3 before staining, was omitted (figure (a)) and introduced (figure (b)).

Figure (a). Lane 1, transferrin; lanes 2-9, as lanes 1-8 in figure (b).

Figure (b). Lanes 1-8, cell envelope fractions from V. anguillarum strains; lane 1, strain 5679; lane 2, strain 4979; lane 3, strain 2981; lane 4, strain 827; lane 5, strain 91079; lane 6, strain 1197; lane 7, strain 636 and lane 8, strain 775; lane 9, transferrin.

(a)

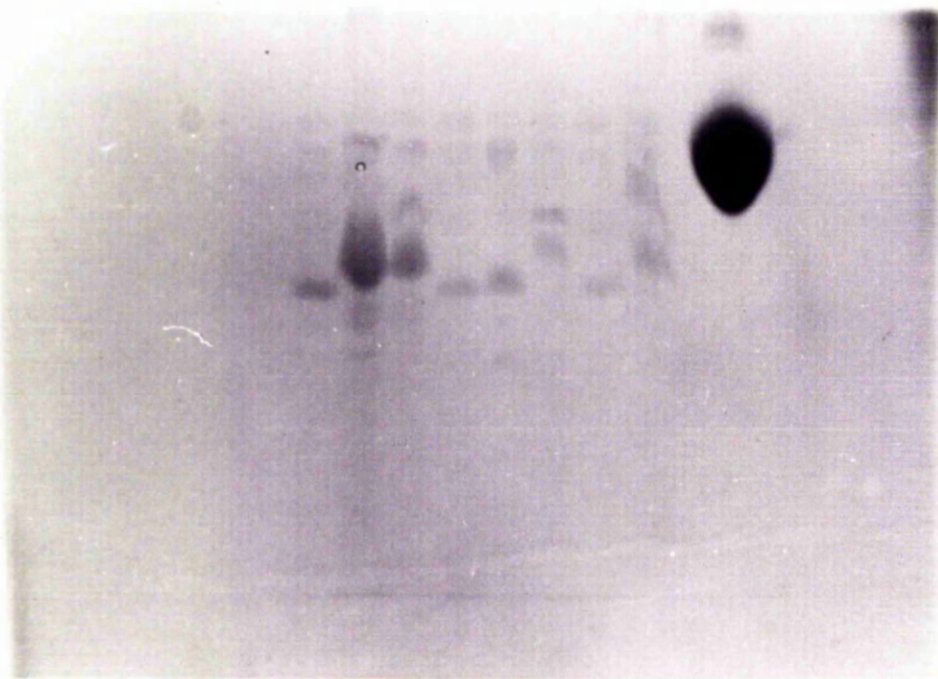
120



1 2 —————> 9

(b)

1 2 3 4 5 6 7 8 9



Although this additional step appeared to make the staining procedure more sensitive for the detection of iron-binding proteins it may also have resulted in non-specific binding. Therefore, no advantage was found in the use of this stain. However, these preliminary experiments did indicate the presence of common iron-binding protein(s) in all the eight V. anguillarum strains tested with a molecular weight of 70-80kD (Figure 25(b)).

4.4 Detection of ^{59}Fe -labelled Proteins From Whole Cell Preparations

When soluble extracts of V. anguillarum cells cultured in either NBS or NBST media were compared by SDS-PAGE with coomassie blue staining no differences in the protein bands were visible (Figure 26). Therefore, in an attempt to detect iron-binding proteins, V. anguillarum strains 775 and NCMB6 were incubated with $0.1\text{mCi } ^{59}\text{FeCl}_3$ for 1h at 25°C in NBS or NBST media before analysis of cellular proteins by SDS-PAGE. After staining with coomassie blue the gel was dried for autoradiography.

Although the 68-79kD envelope proteins previously detected and associated with iron limitation were not seen, four low molecular weight proteins for V. anguillarum 775 and two low molecular weight proteins for V. anguillarum NCMB6 were visualised on the film as indicated in Figure 27. The functions of these proteins are unknown but they may be intracellular proteins associated with iron metabolism. They appeared to be present when the cells were grown in the presence or absence of transferrin.

5. Detection of Three Siderophore Types by Ten Strains of V. anguillarum

As previous sections have shown, the V. anguillarum strains are

Figure 26. SDS-PAGE of whole cell preparations of V. anguillarum strains 775 and NCMB6.

Soluble extracts of V. anguillarum strains 775 and NCMB6 cultured in either NBS, NBST or NBST + FeCl₃ were separated by SDS-PAGE and protein bands visualised with coomassie blue staining.

lane 1, V. anguillarum strain NCMB6, cultured in NBST + FeCl₃;

lane 2, V. anguillarum strain NCMB6, cultured in NBS;

lanes 3 and 4, V. anguillarum strain NCMB6, cultured in NBST;

lane 5, V. anguillarum strain 775, cultured in NBST + FeCl₃;

lane 6, V. anguillarum strain 775, cultured in NBS;

lanes 7 and 8, V. anguillarum strain 775, cultured in NBST.

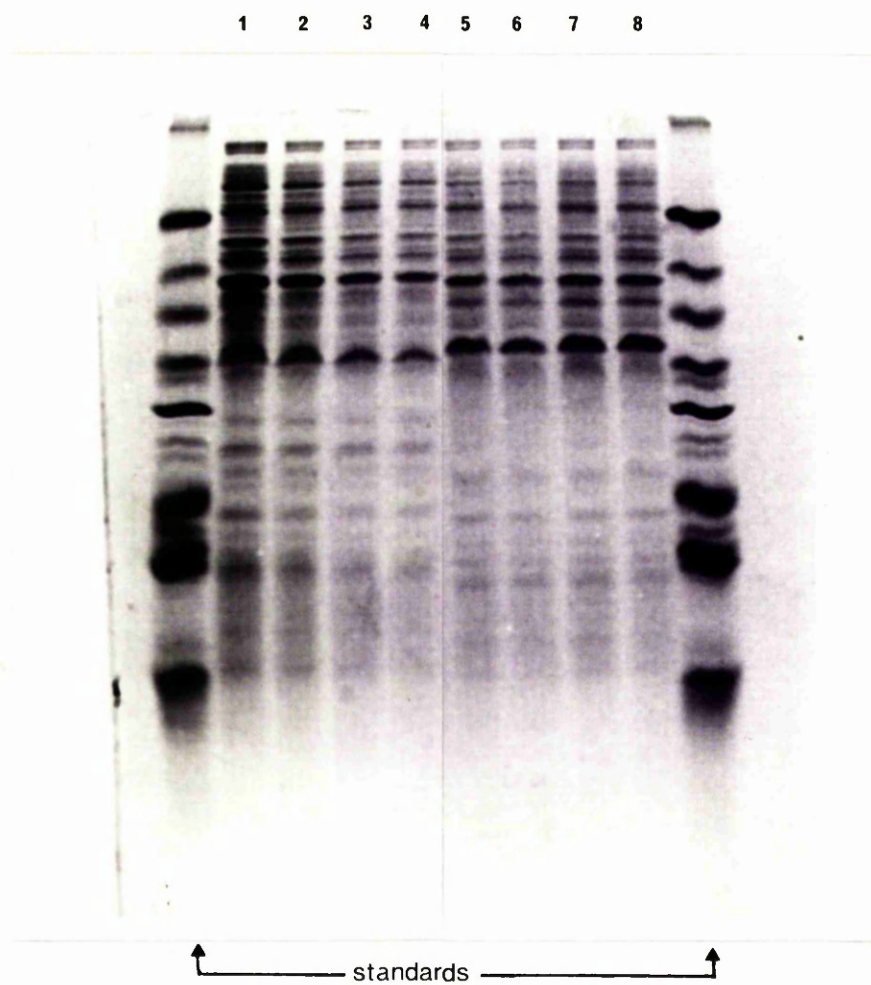
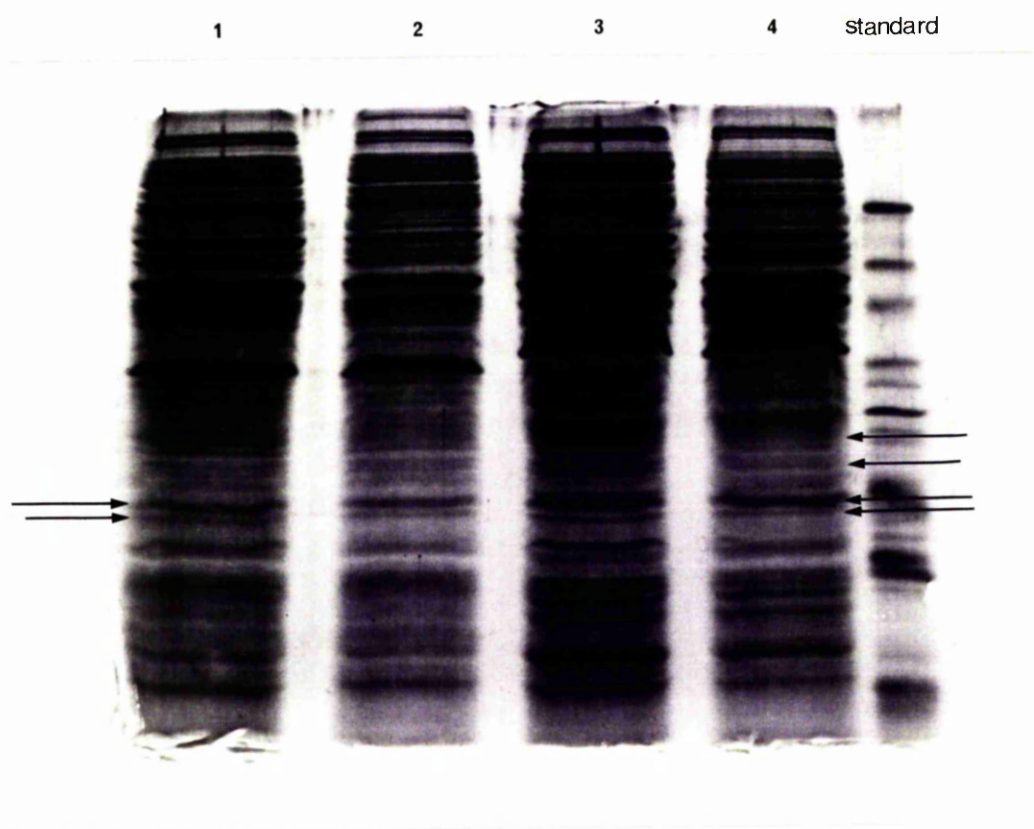


Figure 27. SDS-PAGE of whole cell preparations of V. anguillarum labelled with $^{59}\text{FeCl}_3$.

V. anguillarum strains 775 and NCMB6, cultured in either NBS or NBST, were incubated with $0.1 \text{ mCi } ^{59}\text{Fe}$ for 1h at 25°C and soluble extracts of cells were separated on SDS-PAGE and visualised with coomassie blue stain. Detection of ^{59}Fe -labelled proteins by autoradiography showed two low molecular weight proteins for V. anguillarum strain NCMB6 (lane 1, soluble extract of cells cultured in NBS; lane 2, soluble extract of cells cultured in NBST) and four low molecular weight proteins for V. anguillarum strain 775 (lanes 3 and 4, as lanes 1 and 2 respectively).

The positions of these proteins are indicated by the arrows on either side of the SDS-PAGE.

Autoradiograph could not be photographed.



capable of producing more than one envelope protein under iron-limiting conditions and this suggests that V. anguillarum may possess two or more independent iron-uptake systems, encoded on the chromosome or on a plasmid.

The second component of an iron-uptake system is usually an extracellular, low molecular weight siderophore such as the phenolate-type siderophore, anguibactin, produced by V. anguillarum 775 and encoded on the plasmid pJM1 (Actis et al., 1986). The production of siderophore-types by the ten V. anguillarum strains was therefore investigated in three different iron-limiting media, to confirm the presence of anguibactin and other possible siderophores.

5.1 Determination of Optimum Conditions for Siderophore Production

To determine the optimum conditions for detection of siderophore production by the ten strains of V. anguillarum, all strains were grown in NBS or NBST media. Preliminary experiments used direct inoculation from a plate culture of bacteria into NBS or NBST media and 2ml samples were removed from a 50ml shake flask culture after 24, 48 and 72h of growth at 25°C. The presence of phenolate- or hydroxamate-type siderophores in culture supernate samples was determined by the Arnow Assay and the Csaky test (or ferric perchlorate test) respectively. When negative results were obtained after 72h growth in NBST medium, supernate samples were lyophilised, concentrated 10 fold and retested.

Under these conditions phenolate-type siderophores were detected in culture supernate from only 3 strains of V. anguillarum. After lyophilisation, a positive phenolate reaction was found in 8 of the strains (table 13). Hydroxamate-type siderophores were not detected for any of the strains. No siderophores were detected when the strains were grown in NBS medium.

Table 13. Phenolate-Type Siderophore Reactions by 10 Strains of
V. anguillarum Grown in Oxoid Nutrient Broth No. 2 plus
1.5% NaCl and Transferrin

<u><i>V. anguillarum</i></u> strain	Cultural Conditions		Cultural Conditions	
	A (a)		B (b)	
	Supernatant	10X Concentrate	Supernatant	10X Concentrate
NCMB6	+	+	+	+
775	+	+	+	+
636	+	+	+	+
827	-	+	+	+
1145	-	+	+	+
1197	-	+	-	+
2981	-	+	-	+
4979	-	+	-	+
5679	-	-	-	+
91079	-	-	-	+

(a) Broth cultures were inoculated directly from plate culture.

(b) Broth cultures were inoculated with $1/100$ volume of starter culture grown for 7h.

In an attempt to increase production of siderophores the 50ml shake flask cultures were inoculated with $1/100$ volume of a starter culture grown for 7h at 25°C. After 72h, a positive phenolate-type siderophore reaction was detected in culture supernate from 6 strains of V. anguillarum and from all ten strains after concentration (table 13). Hydroxamate-type siderophores were not detected in any of the strains of V. anguillarum and no siderophore production was detected when the strains were grown in NBS medium.

5.2 Detection of Phenolate- and Hydroxamate-Type Siderophores by V. anguillarum Grown in Three Different Iron-Limiting Media

Three different iron-limiting media were used to compare siderophore production by V. anguillarum.

(a) Vibrio Minimal Medium plus Transferrin (VMMT)

This minimal medium was chosen for radioactive iron-uptake assay experiments (see later section). When lyophilised culture supernates were tested for the presence of siderophores, all 10 strains gave a positive phenolate-type siderophore reaction (table 14(A)) with results similar to those above (section 5.1).

(b) Tris-Succinate Medium (TSM)

In this medium, succinate is the only carbon source and it has previously been used to induce the production of hydroxamate-type siderophores (Braun, 1981). After 72h growth at 25°C, all 10 strains produced a positive phenolate-type siderophore reaction and in addition 3 strains, V. anguillarum strains NCMB6, 636 and 1445, produced a positive hydroxamate-type siderophore reaction (table 14(B)).

(c) Nutrient Broth + Salt and Desferal (NBS + Desferal)

Desferal was added to NBS as an alternative iron-chelator to transferrin. No phenolate-type siderophore reaction was detected after

Table 14. Siderophore Production by Ten *Vibrio anguillarum* Strains Grown in Three DifferentIron-Limiting Media

Growth Medium	Siderophore Reaction	<u>V. anguillarum strains</u>									
		NCMB6	636	1445	775	1197	4979	91079	827	2981	5679
A. <i>Vibrio</i> Minimal media (VMM)	Phenolate	+	+	+	+	+	+	+	+	+	+
	Hydroxamate	-	-	-	-	-	-	-	-	-	-
B. Tris-Succinate medium (TSM)	Phenolate	+	+	+	+	+	+	+	+	+	+
	Hydroxamate	+	+	+	-	-	-	-	-	-	-
C. Oxoid Nutrient Broth No. 2 plus Desferal	Phenolate	-	-	-	-	-	-	-	-	-	-
	Hydroxamate	+	+	+	+	+	+	+	+	+	+

Phenolate reactions were detected by the colorimetric Arnou Assay.

Hydroxamate reactions were detected by the Csaky Test and Ferric perchlorate test.

72h growth at 25°C, and, although a positive hydroxamate-type siderophore reaction was detected in all culture supernates, uninoculated medium also gave a positive result (table 14(C)).

Therefore siderophore production in V. anguillarum appears to be dependent not only on the type of iron-limiting medium, but also on the iron chelator used to bind any available ferric iron in the culture medium.

5.3 Paper Chromatography of Phenolate-Type Siderophores

To detect phenolate-type siderophore(s) produced by V. anguillarum after growth in NBST for 72h at 25°C, phenolic compounds were extracted from lyophilised culture supernates with ethyl acetate, as outlined in Materials and Methods. Components of the ethyl acetate extract were separated by ascending paper chromatography with a solvent system of 5% ammonium formate and 0.5% formic acid and the dried chromatography paper was viewed under ultraviolet (UV) light before staining with 1% (w/v) FeCl₃ and ammonia vapour. Three strains (V. anguillarum 775, 1197 and 4979) produced two phenolic, iron-binding compounds, whereas the remaining seven strains produced only one (Figures 28, 29 and 30). The iron-binding phenolic compound produced by strains 775, 1197 and 4979 showed a blue fluorescence when viewed under UV, formed a purple/blue complex with iron and migrated with an R_f value of 0.45-0.50. The common phenolic iron-binding compound by all ten strains exhibited a yellow/white fluorescence, formed a pink/lilac complex with iron and migrated with an R_f value of 0.60-0.70 (table 15).

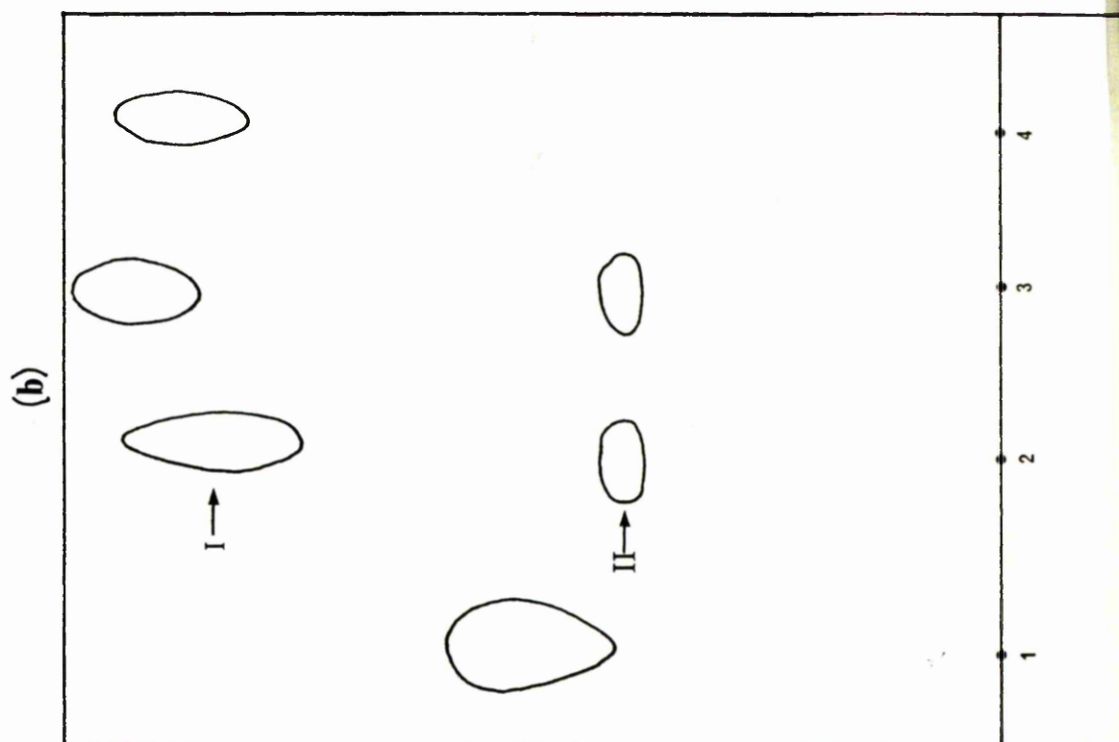
5.4 Paper Chromatography of Hydroxamate-Type Compounds

The hydroxamic compounds produced by V. anguillarum strains

Figure 28. Paper chromatography of ethyl acetate extracts of culture supernates of V. anguillarum strains 775, 4979 and 5679.

Paper chromatography of ethyl acetate extracts of culture supernates of V. anguillarum strains 775, 4979 and 5679 (a) viewed under ultraviolet illumination and (b) after staining with 1% (w/v) FeCl_3 and ammonia vapour. The position of the common iron-binding compound, which showed yellow fluorescence and a pink/lilac complex with iron, and the second iron-binding compound (showing blue fluorescence and a purple/blue complex with iron) are marked I and II respectively.

Lane 1, 2,3-dihydroxybenzoic acid;
lane 2, V. anguillarum strain 775; lane 3, V. anguillarum strain 4979; lane 4, V. anguillarum strain 5679.



(a)

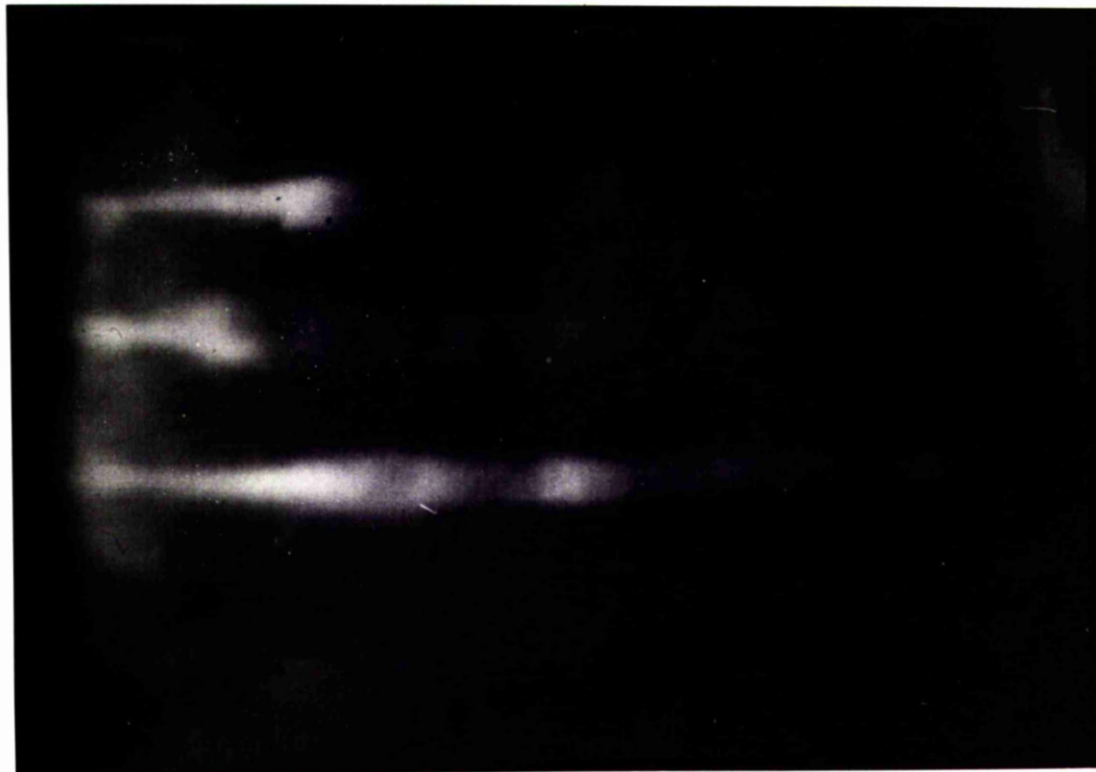


Figure 29. Paper chromatography of ethyl acetate extracts of culture supernates of V. anguillarum strains 636, NCMB6 and 2981.

Paper chromatography of ethyl acetate extracts of culture supernates of V. anguillarum strains 636, NCMB6 and 2981, (a) viewed under ultraviolet illumination and (b) after staining with 1% (w/v) FeCl_3 and ammonia vapour. The position of the common iron-binding compound, which showed yellow fluorescence and a pink/lilac complex with iron is marked I on figure (b).

Lane 1, 2,3-dihydroxybenzoic acid;
lane 2, V. anguillarum strain 636; lane 3, V. anguillarum strain NCMB6; lane 4, V. anguillarum strain 2981.

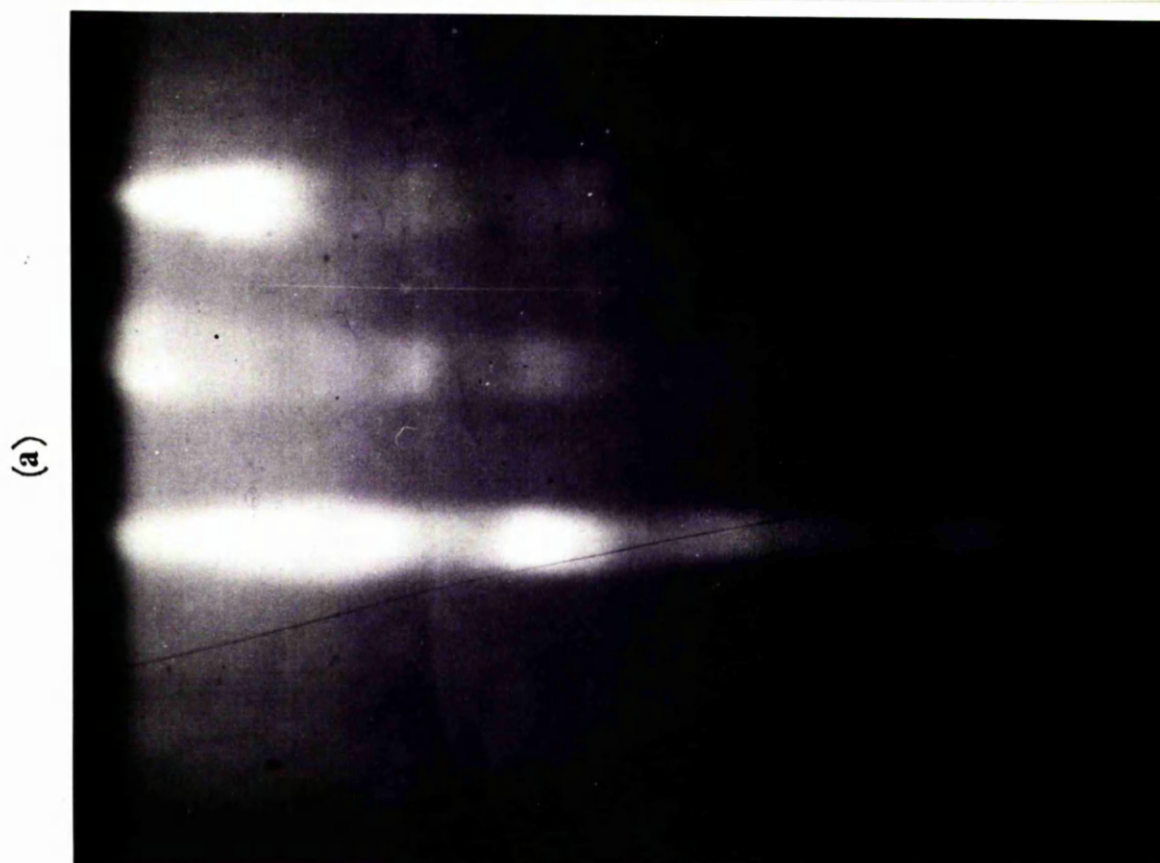
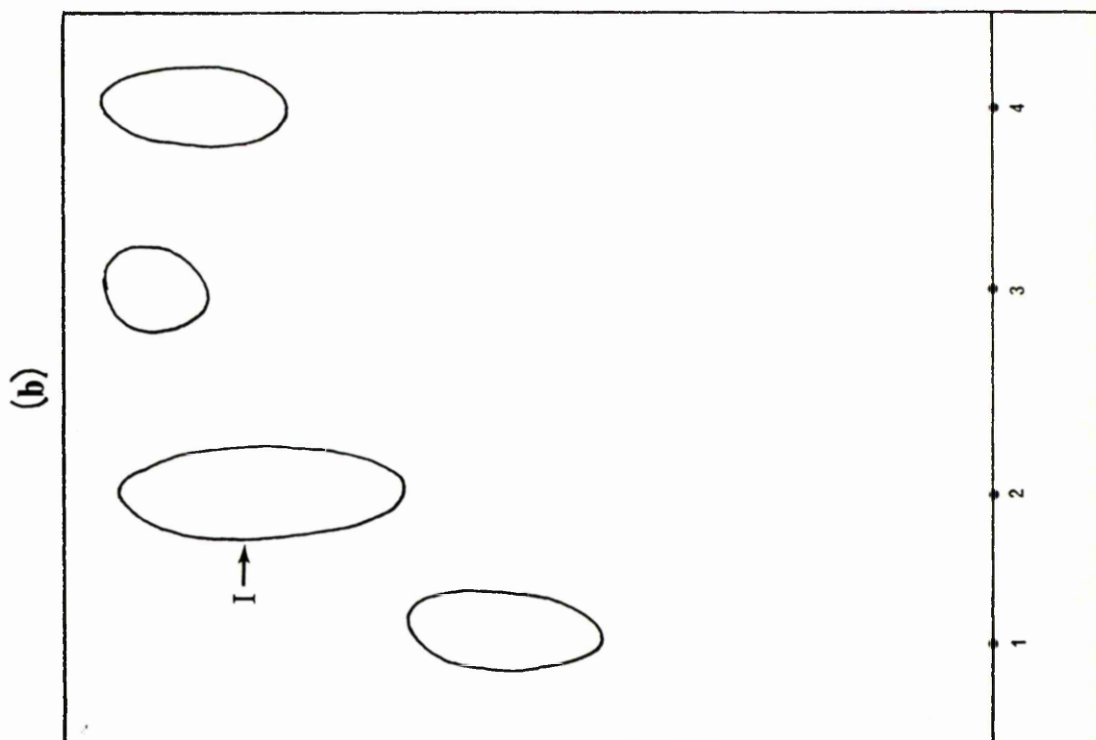
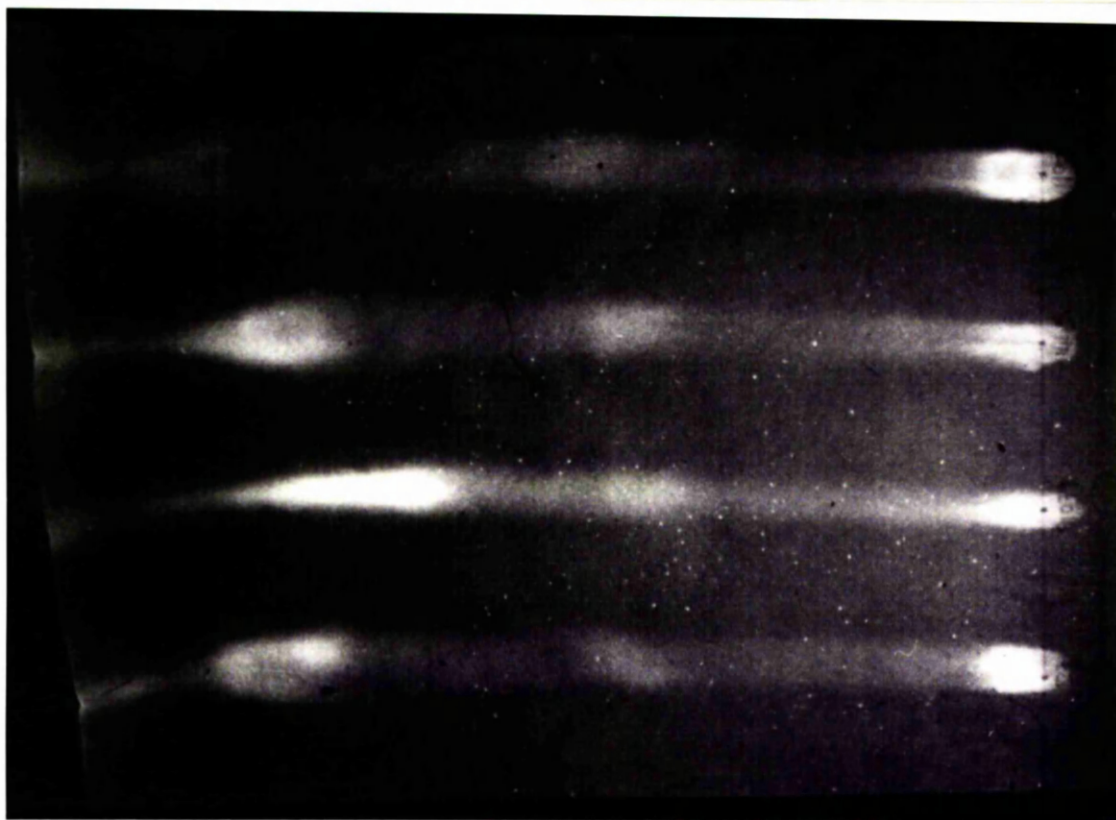


Figure 30. Paper chromatography of ethyl acetate extracts of culture supernates of V. anguillarum strains 827, 91079, 1445 and 1197.

Paper chromatography of ethyl acetate extracts of culture supernates of V. anguillarum strains 827, 91079, 1445 and 1197, (a) viewed under ultraviolet illumination and (b) after staining with 1% (w/v) FeCl_3 and ammonia vapour. The position of the common iron-binding compound, which showed yellow fluorescence and a pink/lilac complex with iron, and the second iron-binding compound (showing blue fluorescence and a purple/blue complex with iron) are marked I and II respectively.

Lane 1. 2,3-dihydroxybenzoic acid; lane 2, V. anguillarum strain 827; lane 3, V. anguillarum strain 91079; lane 4, V. anguillarum strain 1445; lane 5, V. anguillarum strain 1197.

(a)



(b)

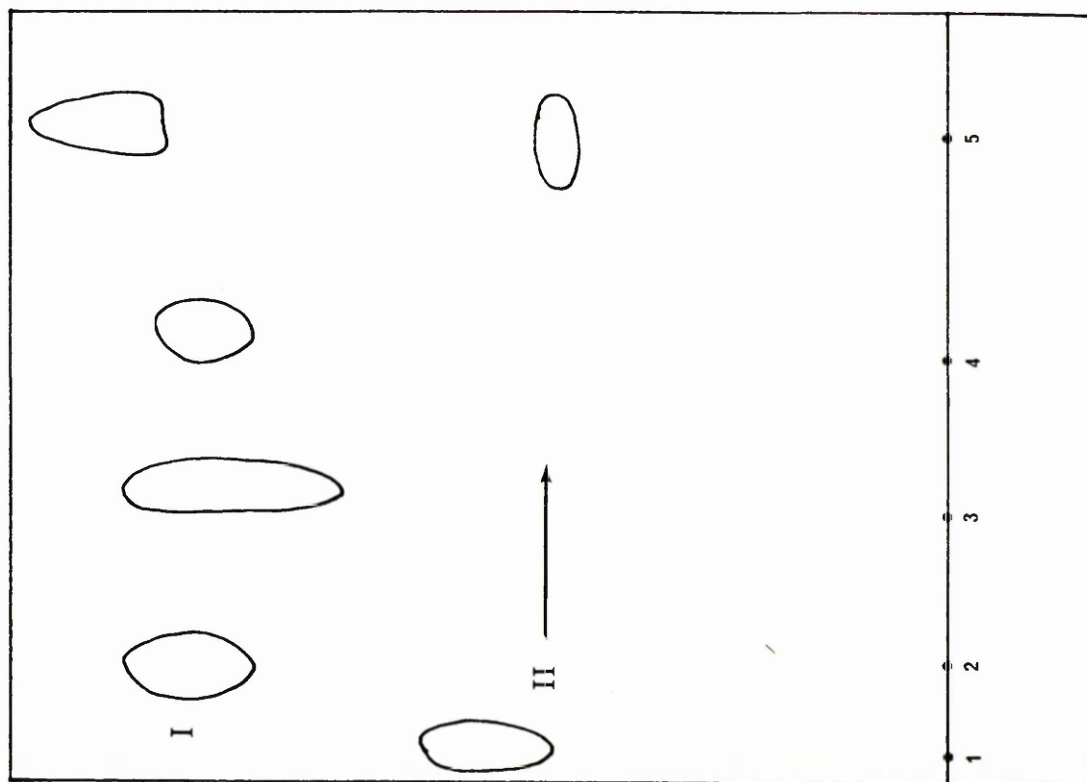


Table 15. Characteristics on Paper Chromatography of Phenolate-Type Compounds Produced by

10 Strains of V. anguillarum

<u>V. anguillarum</u> strain	Type-I (b)			Type-II (c)		
	fluorescence	Iron Complex	Rf Value	fluorescence	Iron Complex	Rf Value
		Colour			Colour (a)	
775	blue	purple/blue	0.451	yellow/white	lilac/pink	0.618
1197	blue	purple/blue	0.420	yellow/white	lilac/pink	0.701
4979	blue	purple/blue	0.402	yellow/white	lilac/pink	0.628
NCMB6	-	-	-	yellow/white	lilac/pink	0.620
91079	-	-	-	yellow/white	lilac/pink	0.650
636	-	-	-	yellow/white	lilac/pink	0.702
827	-	-	-	yellow/white	lilac/pink	0.635
1445	-	-	-	yellow/white	lilac/pink	0.600
2981	-	-	-	yellow/white	lilac/pink	0.675
5679	-	-	-	yellow/white	lilac/pink	0.625

(a) Colour complex detected only in the presence of FeCl_3 and ammonia vapour.

(b) Type I represents the plasmid-associated phenolate-type compound.

(c) Type II represents the common phenolate-type compound.

NCMB6, 636 and 1445 when grown in TSM medium were detected on paper chromatography using a solvent system of n-butanol-water-acetic acid (60:15:25 v/v) and culture supernate concentrated by lyophilisation. The iron-binding hydroxamic-compound showed a dark blue/purple fluorescence under UV, formed a red/brown complex with iron and migrated with an R_f value of 0.35-0.44. Aerobactin, which was used as a reference standard, migrated with an R_f value of 0.50 (Table 16).

A positive hydroxamate-type siderophore reaction was also detected when strains were grown in NBS + desferal. When lyophilised culture supernate samples were separated on paper chromatography the only iron-binding spots visualised after staining with iron migrated with R_f values corresponding to those of desferal (table 17). Therefore, although V. anguillarum does not produce a siderophore, it does appear to obtain iron from desferal.

5.5 The Detection of Phenolate-Type Siderophores of V. anguillarum strains 775, 4979 and 1197 in TSM Plus Transferrin

V. anguillarum strains 775, 1197 and 4979, grown in TSM, produced only one iron-binding compound which corresponded to the phenolate compound common to all ten strains (table 16). This suggested that the second phenolate compound was not produced when these strains were grown in TSM. However, when transferrin ($100 \mu\text{gml}^{-1}$) was added to TSM, the second phenolate, iron-binding compound was detected in culture supernates of the three strains. This is demonstrated in figure 31 with ethyl acetate extracts of V. anguillarum 775 culture supernate grown in TSM and TSMT. The chromatography paper was viewed under UV light before staining with iron and ammonia vapour. These results indicated that the production of the ^{Type I} phenolate iron-binding

Table 16. Characteristics on Paper Chromatography of Hydroxamate-Type Compounds Produced by

Three Strains of *V. anguillarum* Grown in Tris Succinate Medium

<i>V. anguillarum</i> strain	Type-A (a)		Type-B (b)	
	fluorescence	Iron Complex Colour	fluorescence	Iron Complex Colour
NCMB6	dark purple	brown	white/yellow	(c) 0.503
636	dark purple	brown	white/yellow	(c) 0.406
1445	dark purple	brown	white/yellow	(c) 0.444
4979 (d)	-	-	white/yellow	(c) 0.444
Aerobactin (e)	-	brown	-	-
(a) Type A	represents the hydroxamate-type compound.			
(b) Type B	represents the common phenolate-type compound.			
(c) Not determined	due to heavily coloured background.			
(d)	Strain 4979 was used as a control not producing a positive hydroxamate reaction.			
(e)	Aerobactin was a standard hydroxamate-type siderophore.			

Table 17. Characteristics on Paper Chromatography of the Hydroxamate
Compounds Produced by *V. anguillarum* After Growth in Oxoid
Nutrient Broth plus 1.5% NaCl and Desferal

	<u><i>V. anguillarum</i></u>		Desferal	
	culture supernate			
Colour Complex with Iron	dark blue	dark blue	dark blue	dark blue
R _f Value*	0.840([±] 0.02)	0.930([±] 0.01)	0.80	0.930

*The R_f values are mean [±] standard error of the mean for n=10.

Figure 31. Paper chromatography of ethyl acetate extracts of culture supernates of V. anguillarum strain 775 cultured in TSM and TSMT.

Paper chromatography of ethyl acetate extracts of culture supernates of V. anguillarum strain 775 cultured in either TSM or TSMT (a) viewed under ultraviolet illumination and (b) after staining with 1% (w/v) FeCl_3 and ammonia vapour.

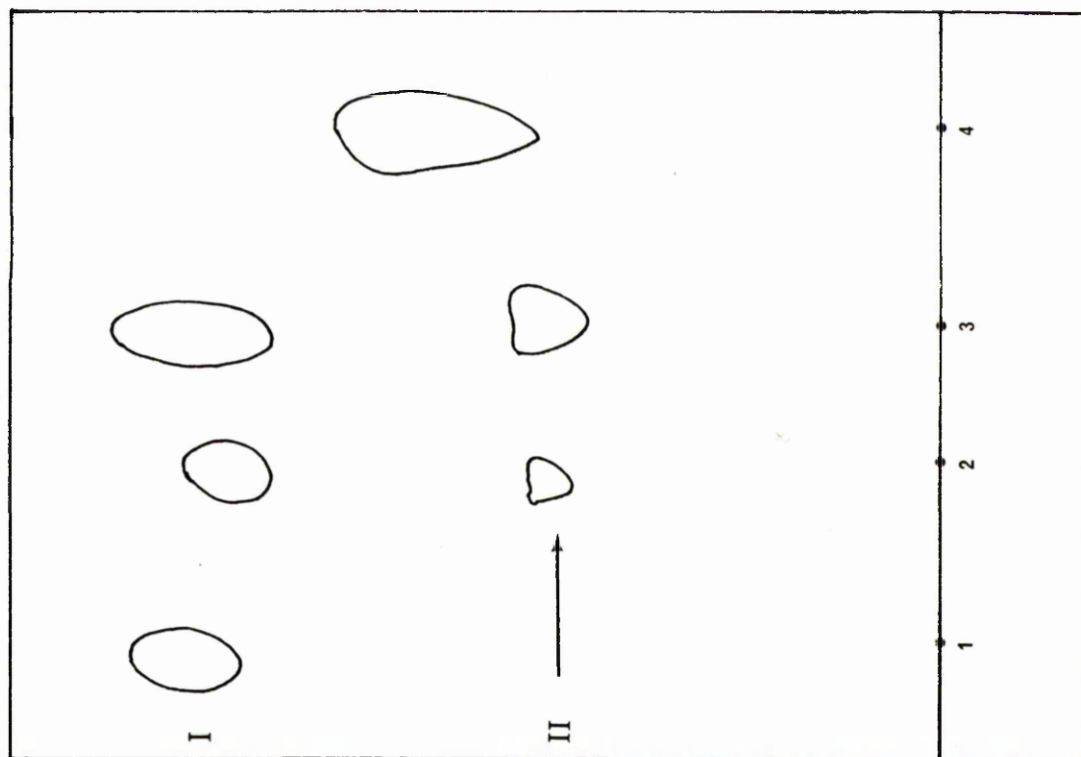
Lane 1, V. anguillarum strain 775 cultured for 72h in TSM; lane 2, V. anguillarum strain 775 cultured for 24h in TSMT; lane 3, V. anguillarum strain 775 cultured for 72h in TSMT; lane 4, 2,3-dihydroxybenzoic acid.

The position of the common phenolate iron-binding compound I and the second phenolate iron-binding compound II are marked on figure (b).

(a)



(b)



compound by the three strains depended on the presence of transferrin. Since the three strains also contained a plasmid of similar molecular weight, the production of the second phenolic compound appeared to be associated with carriage of the 45-50mD plasmids.

The properties of the ten strains of V. anguillarum when grown under conditions of iron-limitation are summarised in table 18.

6. Determination of Iron-Uptake Ability Using Radioactive Iron (^{55}Fe)

A radioactive iron-uptake assay was adapted from the method of Crosa and Hodges (1981) as a basis to determine the following:

- (a) Which of the ten strains of V. anguillarum was the most efficient in removing iron from VMM.
- (b) Which of the three iron-binding compounds produced by the V. anguillarum strains had the highest relative affinity for iron.

6.1 Determination of the Iron-Uptake Ability

The ability of the ten strains of V. anguillarum to take up radioactive iron ($^{55}\text{FeCl}_3$) after growth under iron-limitation (as outlined in Materials and Methods) was investigated. The logarithm of time was plotted with the measured radioactivity (cpm) for each strain (Figures 32, 33, 34, 35 and 36). In all cases a straight line was fitted by regression analysis and the slopes were calculated from each line. In table 19 the V. anguillarum strains are graded in descending order of their ability to take up ^{55}Fe (using the slope of each line as a rate of iron-uptake).

V. anguillarum strains 775 and 1197 appeared to be the most efficient and it is interesting to note that these strains produced two cell envelope proteins associated with iron-limitation, and two

Table 18. Comparison of Iron-Binding Compounds and Cell Envelope
Proteins Associated with Iron-Limitation and Plasmid
Content in 10 Strains of *V. anguillarum*

<u><i>V. anguillarum</i></u>	Iron-Binding Compounds	Envelope Proteins	Plasmid
strain	Produced During	Associated with	Content
	Iron-Limitation	Iron-Limitation	
775	Phenolate I, Phenolate II	79kD 72.5kD	+
1197	Phenolate I, Phenolate II	78kD 73kD	+
4979	Phenolate I, Phenolate II	73kD	+
5679	Phenolate II	78kD 73kD	-
827	Phenolate II	71kD	-
91079	Phenolate II	72kD	-
NCMB6	Hydroxamate I, Phenolate II	69kD	-
636	Hydroxamate I, Phenolate II	71kD	-
1445	Hydroxamate I, Phenolate II	73kD 72kD 68kD	-

Figure 32. Uptake of ^{55}Fe by washed cell suspensions of V. anguillarum strains 775 and NCMB6 in VMM. The straight line was fitted by Regression Analysis.

□ — □ V. anguillarum strain NCMB6
△ — △ V. anguillarum strain 775

Lines fitted by regression analysis are shown with solid symbols.

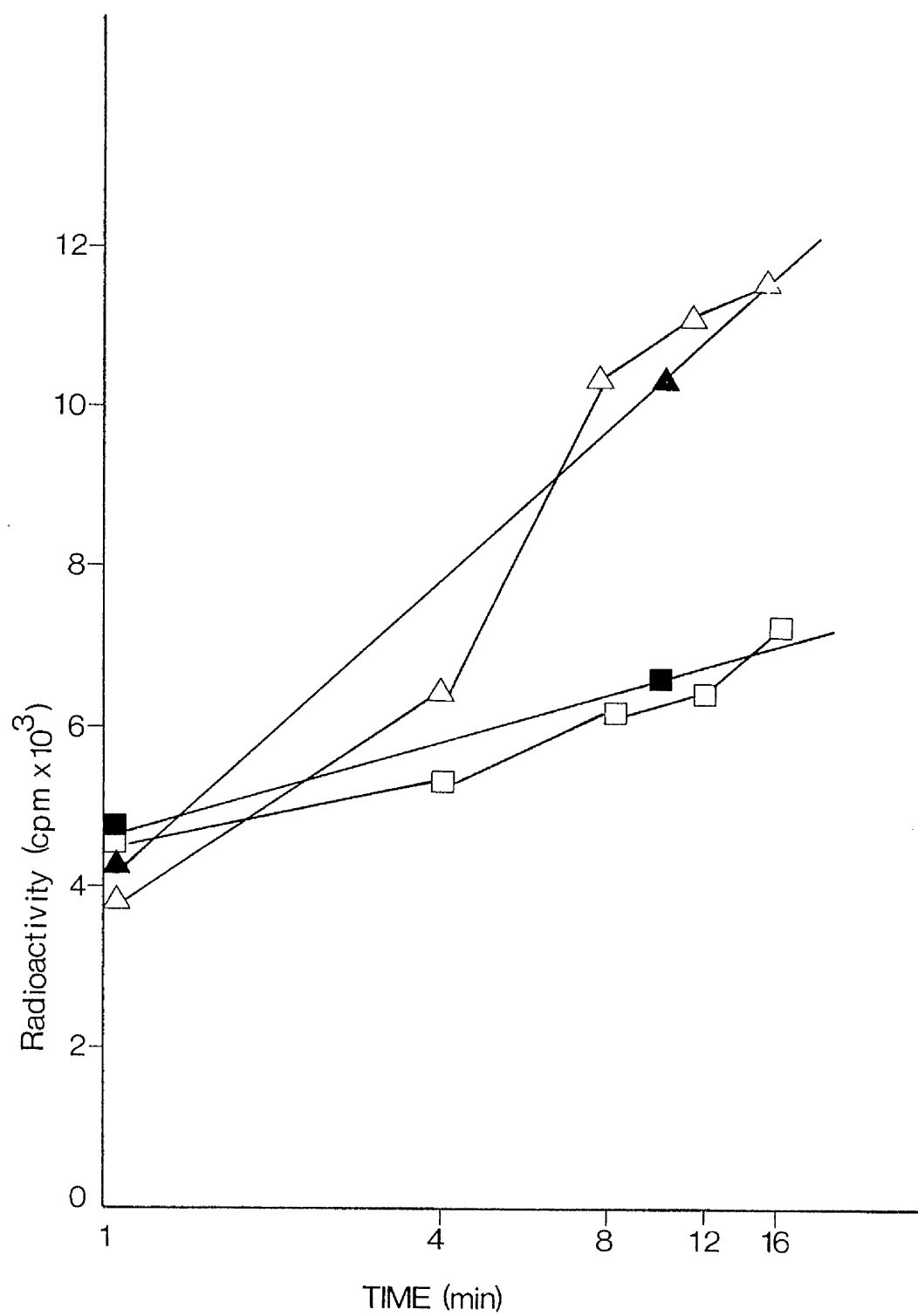


Figure 33. Uptake of ^{55}Fe by washed cell suspensions of V. anguillarum strains 4979 and 636 in VMM. The straight line was fitted by Regression Analysis.

□ — □ V. anguillarum strain 4979
△ — △ V. anguillarum strain 636

Lines fitted by regression analysis are shown with solid symbols.

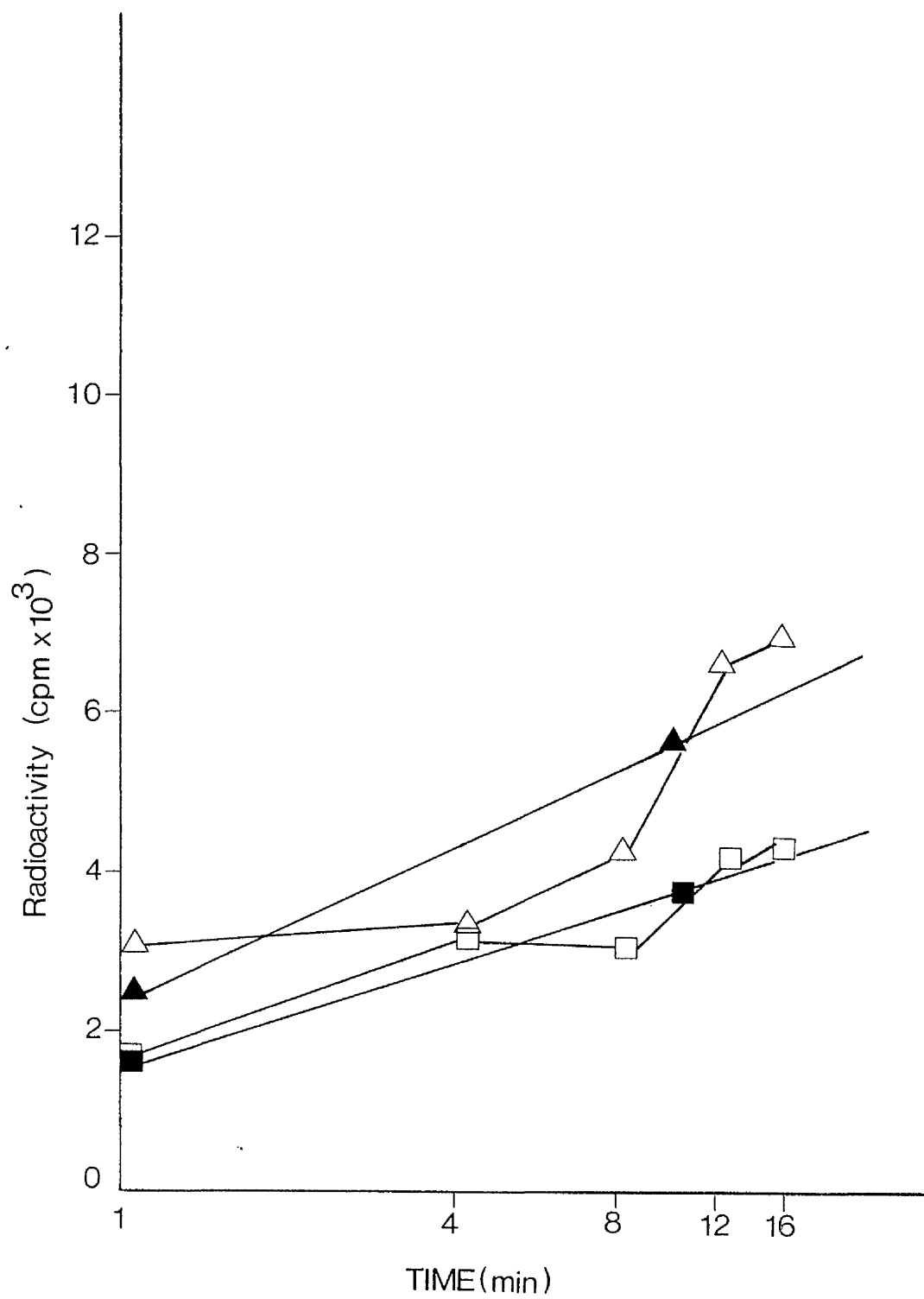


Figure 34. Uptake of ^{55}Fe by washed cell suspensions of V. anguillarum strains 5679 and 827 in VMM. The straight line was fitted by Regression Analysis.

□ — □ V. anguillarum strain 5679
△ — △ V. anguillarum strain 827

Lines fitted by regression analysis are shown with solid symbols.

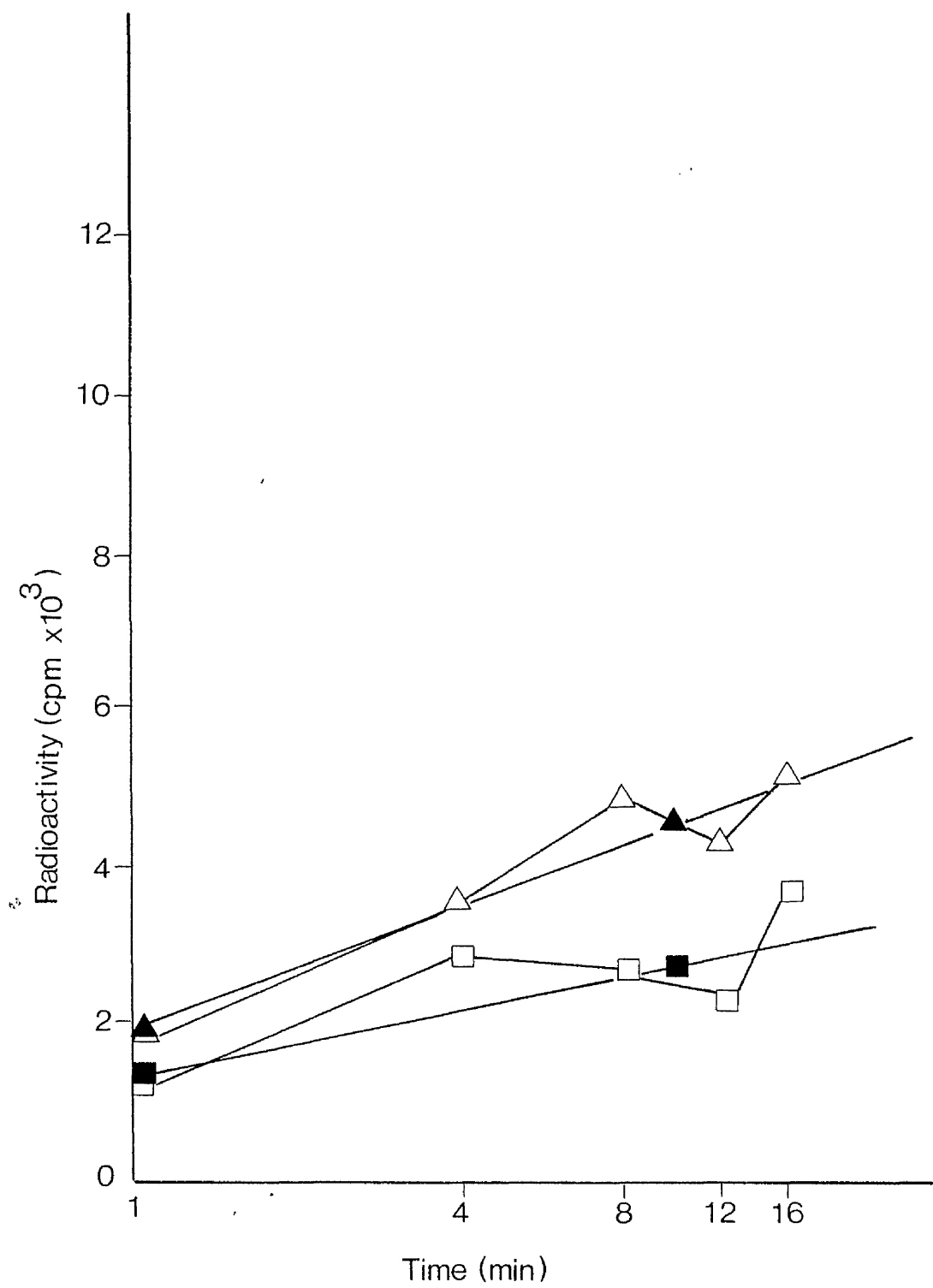


Figure 35. Uptake of ^{55}Fe by washed cell suspensions of V. anguillarum strains 1445 and 1197 in VMM. The straight line was fitted by Regression Analysis.

□ — □ V. anguillarum strain 1445
△ — △ V. anguillarum strain 1197

Lines fitted by regression analysis are shown with solid symbols.

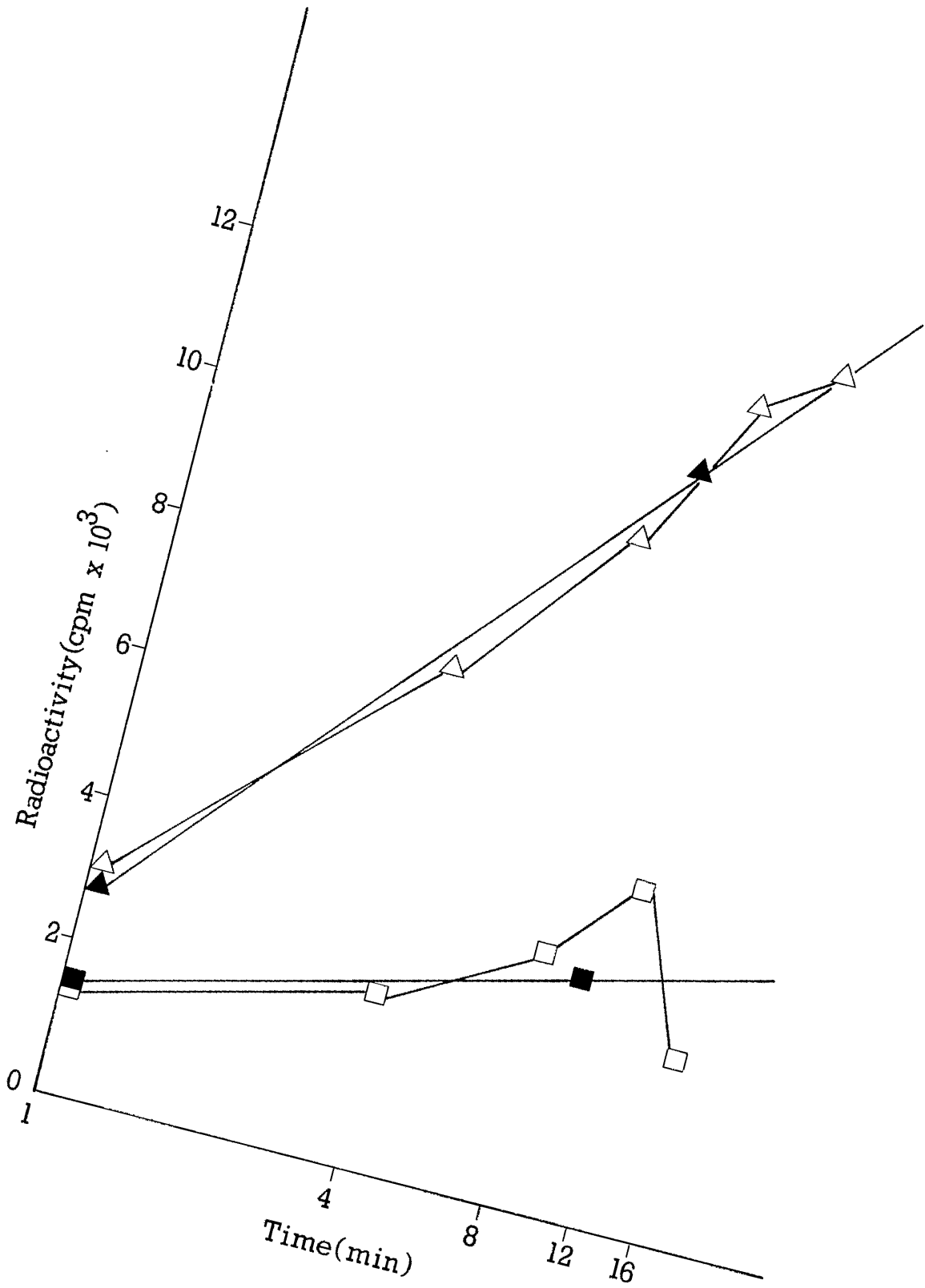


Figure 36. Uptake of ^{55}Fe by washed cell suspensions of V. anguillarum strains 91079 and 2981 in VMM. The straight line was fitted by Regression Analysis.

□ — □ V. anguillarum strain 91079
△ — △ V. anguillarum strain 2981

Lines fitted by regression analysis are shown with solid symbols.

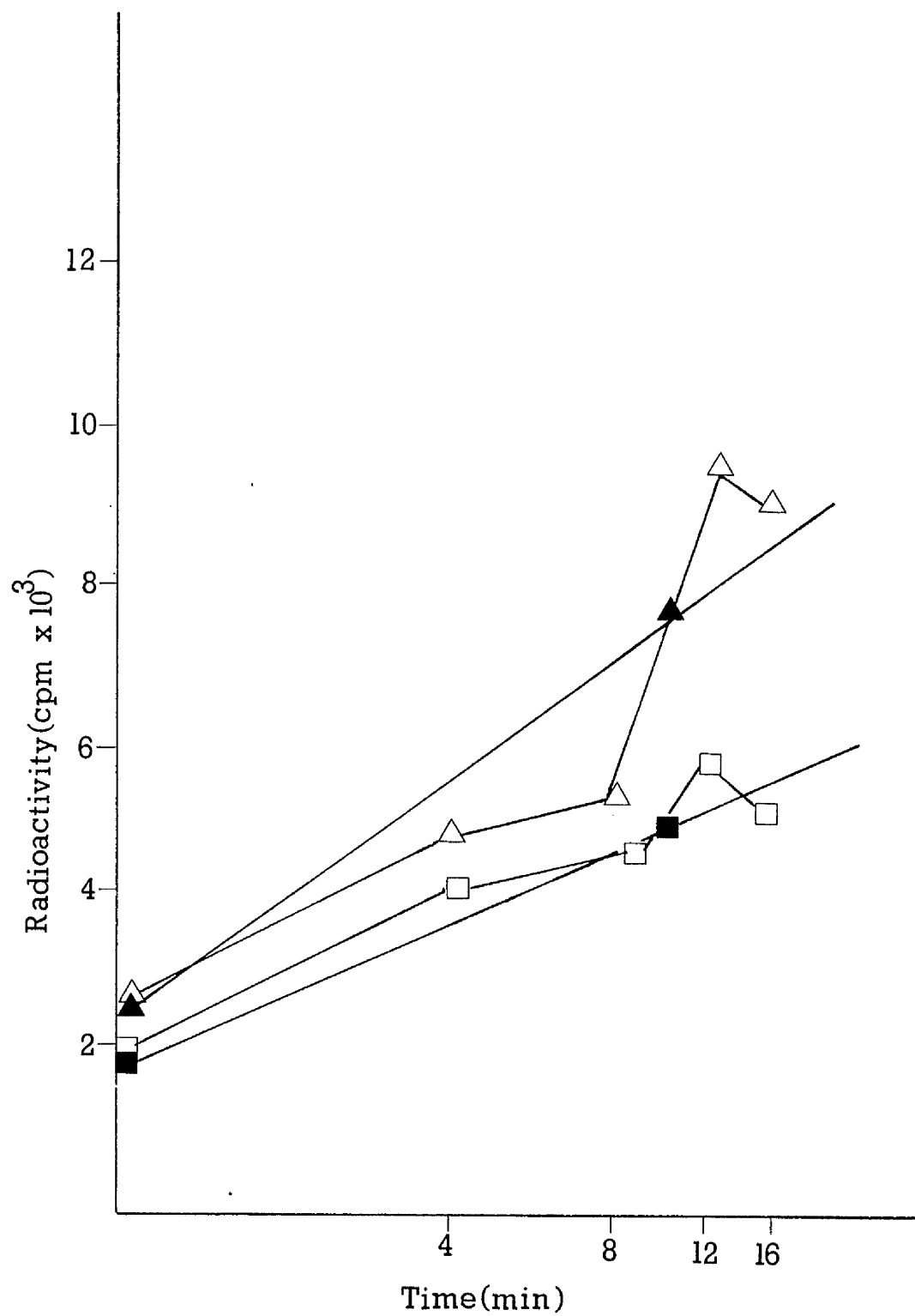


Table 19. The Uptake of ^{55}Fe by 10 Strains of V. anguillarum

<u>V. anguillarum</u>	Iron Uptake (a)
strain	($\times 10^3$) (slope)
1197	7.644 (\pm 0.027)
775	6.493 (\pm 0.032)
2981	5.566 (\pm 0.099)
636	3.250 (\pm 0.135)
91079	2.806 (\pm 0.049)
827	2.626 (\pm 0.045)
4979	2.246 (\pm 0.026)
NCMB6	1.978 (\pm 0.059)
1445	0.753 (\pm 0.113)
5679	1.304 (\pm 0.215)

(a) Iron-Uptake was determined from the slope of the theoretical straight line calculated from Regression Analysis of the radioactive counts per minute (cpm) plotted against \log_{10} (time).
 The values are mean \pm standard error of the mean for $n=3$.

phenolic iron-binding compounds, one associated with carriage of a plasmid (40-50mD).

6.2 Determination of the Relative Affinities for Iron of the Three Siderophores Produced by V. anguillarum strains

Since there appeared to be at least two independent iron-uptake systems within the strains of V. anguillarum, the relative affinity for iron of the three iron-binding compounds produced in different media was measured to grade the iron-uptake systems in the efficiency of iron-uptake.

The ^{55}Fe -Uptake assay was modified to determine whether the production of different compounds by strains 775 and NCMB6, representing producers of the second phenolic iron-binding compound and hydroxamic compound respectively, gave a competitive advantage over strains producing only the common phenolic iron-binding compound. V. anguillarum strains 775 and NCMB6 were grown in three different media and the culture supernates were compared for their ability to inhibit uptake of ^{55}Fe by all other strains; such competitive inhibition is demonstrated in figure 37.

Culture supernates of V. anguillarum strain 775, which produced two phenolic compounds when grown in VMMT medium, inhibited the uptake of ^{55}Fe by strains which produced only the common phenolic compound in VMMT. V. anguillarum strain NCMB6 produced only the common phenolic compound in VMMT and such culture supernates did not inhibit ^{55}Fe uptake by any strain (Table 20(a)).

When V. anguillarum NCMB6 was grown in TSM, both the hydroxamic compound and common phenolic compound were produced, whereas V. anguillarum 775 only produced the common phenolic compound. In

Figure 37. Inhibition of uptake of ^{55}Fe by washed cell suspensions of V. anguillarum strains 775 and NCMB6 by culture supernates of these strains grown in VMMT.

Washed cells of V. anguillarum strain NCMB6
□ — □ suspended in culture supernate of
V. anguillarum strain 775

Washed cells of V. anguillarum strain 775
△ — △ suspended in culture supernate of
V. anguillarum strain NCMB6.

Lines fitted by regression analysis are shown with solid symbols.

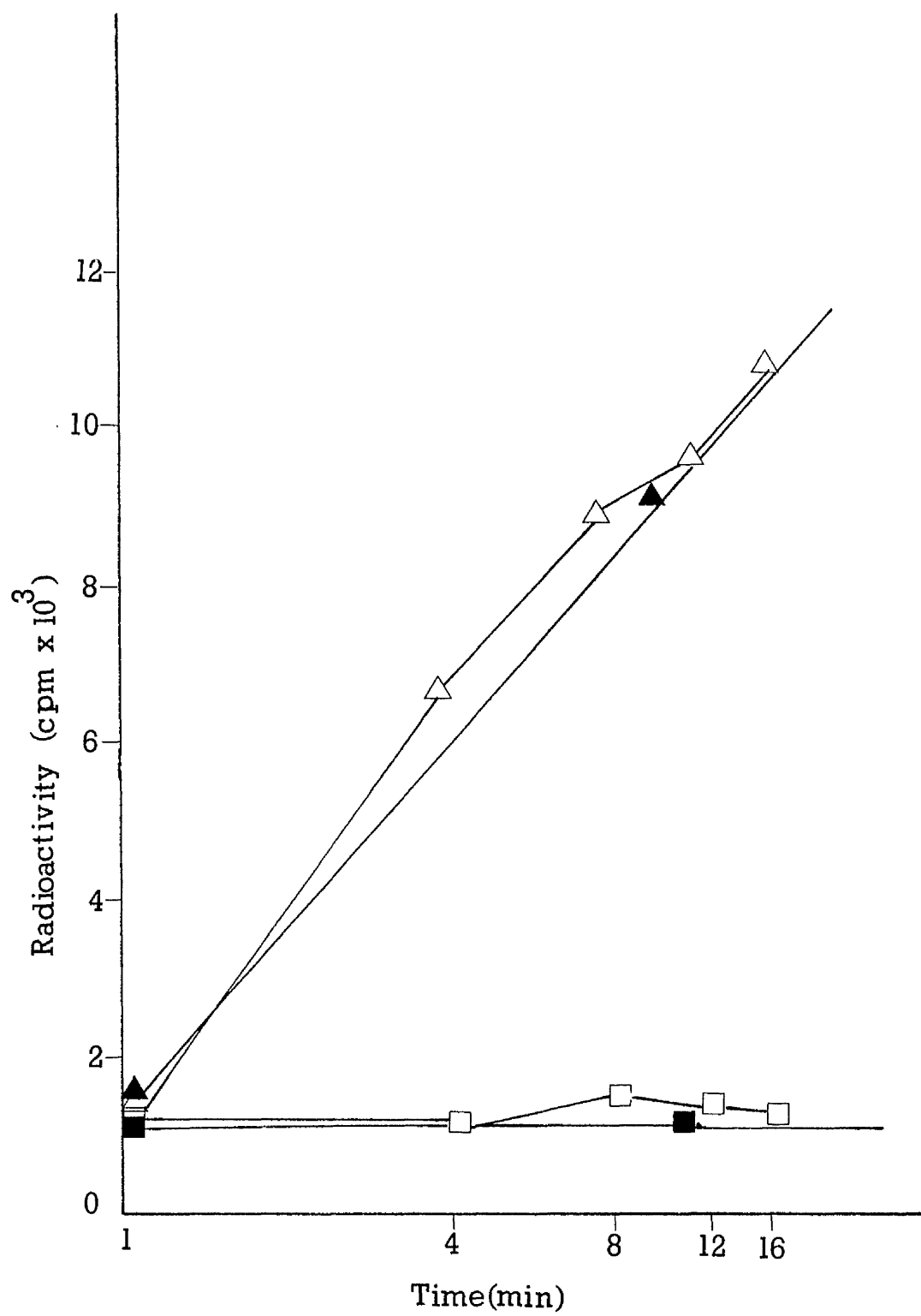


Table 20. Inhibition of the Uptake of ^{55}Fe by Washed Suspensions of V. anguillarum cells by CultureSupernates of V. anguillarum strains 775 and NCMB6

Growth Medium	culture supernate	Uptake of ^{55}Fe by Cells of strain:									
		NCMB6	636	1445	775	1197	4979	91079	827	2981	5679
A. VMM	NCMB6	-	-	-	-	-	-	-	-	-	-
	775	+	+	+	-	-	-	+	+	+	+
B. TSM	NCMB6	-	-	-	+	+	+	+	+	+	+
	775	-	-	-	-	-	-	-	-	-	-
C. TSMT	NCMB6	-	-	-	-	-	-	+	+	+	+
	775	+	+	+	-	-	-	+	+	+	+

+ indicates that inhibition of the ^{55}Fe uptake occurred in the presence of the culture supernate.

- indicates that ^{55}Fe was taken up readily by the cells and no inhibition occurred.

these experiments, only NCMB6 culture supernate inhibited ^{55}Fe uptake by those strains producing only the common phenolic compound, but not with the strains producing the hydroxamic compound and common phenolic compound (Table 20(b)).

In the third medium, both V. anguillarum strains 775 and NCMB6 produced two iron-binding compounds, therefore allowing the grading of the compounds in their relative affinity for iron. As seen in Table 20(c) V. anguillarum 775 culture supernate inhibited the uptake of ^{55}Fe by any strain not producing the second phenolic compound, suggesting that this compound has the highest affinity for iron. Culture supernates of V. anguillarum NCMB6 inhibited the uptake of ^{55}Fe uptake by any strain only producing the common phenolic compounds, but not the strains producing the two phenolic compounds.

Since these results suggest the presence of three independent iron-uptake systems in V. anguillarum, the three iron-binding compounds could tentatively be classified as siderophores. These siderophores were therefore graded according to their relative affinities for iron in the order: second phenolate-type siderophore > Hydroxamate-type siderophore > common phenolate-type siderophore.

6.3 Inhibition of ^{55}Fe -Uptake by the Iron Chelators Aerobactin and Desferal

To determine if other bacterial iron chelators had any effect on the uptake of iron by V. anguillarum, aerobactin or desferal were initially added to VMM in a final concentration of 500 $\mu\text{g}/\text{ml}$ and then to culture supernates of V. anguillarum strains grown in NBST or TSM. These inhibition assays were used to determine the ability of aerobactin or desferal to inhibit uptake of ^{55}Fe by V. anguillarum. In all cases iron-uptake was inhibited by the presence of both chelators.

7. Isolation of Siderophores from the Kidney and Spleen of
Rainbow Trout (*Salmo gairdneri*) Infected with *V. anguillarum*

Although the production of three siderophores had been demonstrated in vitro, it was of interest to determine which, if any, of the siderophores could be detected in vivo during experimental infections of rainbow trout (*Salmo gairdneri*).

7.1 Determination of Optimum Conditions to Induce Experimental
Vibriosis in Rainbow Trout

Preliminary experiments on rainbow trout were carried out at 10°C, but even when 10⁹ *V. anguillarum* 775 cells were injected, no outward signs of disease were visible and the fish survived for at least 21 days after infection. Vibriosis did occur when the water temperature in aquarium tanks was raised to 15-17°C, and all subsequent experiments were carried out at this temperature. Vibriosis was observed 1-4 days after injection and outward signs of the disease included haemorrhagic skin lesions, internal bleeding and uncontrolled swimming movements. In some cases the dorsal fin was rotted and covered in a thick slime layer. The fish were killed when the acute disease was visible, frozen immediately and stored at -20°C for later bacteriological examination and analysis for the presence of bacterial siderophores in spleen and kidney samples. For the spleen samples, only 1 or 2 were recovered from each group of experiments as spleen liquefaction commonly occurred during infection.

For *V. anguillarum* strain 775, inocula from 10² to 10⁹ cells per fish were given to determine the effect of inoculum size on yields of bacteria in the liver and spleen, and to determine the effect of freezing on the recovery of bacteria from tissue samples. All fish injected

with 10^9 or 10^4 bacteria died 1 or 2 days after infection and before substantial bacterial multiplication and siderophore production could be detected in kidney samples (table 21 and figure 38). Therefore in subsequent experiments an inoculum of 10^2 cells was used.

To determine the effect of freezing on the recovery of bacteria from tissues, fish injected with 10^4 cells of V. anguillarum 775 were dissected immediately after killing and the recovery of bacteria was compared to the number found in fish which had been frozen after death (table 22). Recoveries were very similar and subsequently fish were stored at -20°C until each experiment had been completed.

7.2 The Isolation of Siderophores from Kidney and Spleen Homogenate Samples of Infected Rainbow Trout

V. anguillarum strains 775, NCMB6 and 91079 were chosen as representative producers of different classes of siderophores. All produce a common phenolate-type siderophore; in addition strain 775 produces a second phenolate siderophore and strain NCMB6 produces a hydroxamate-type siderophore when grown in TSM.

From an initial inoculum of 10^2 bacteria (5 fish injected per V. anguillarum strain), between 5×10^5 and 5×10^6 bacteria were recovered from the spleen or kidney (Table 23), indicating that at least 10^3 to 10^4 fold replication had occurred during infection.

When supernates of homogenates of kidney and spleen from infected and uninfected fish were analysed for soluble siderophores, all strains produced at least one siderophore during infection of fish (figure 39). In extracts of the kidneys of fish infected with V. anguillarum strain 775, both the common phenolate-type siderophore and the second phenolate-type siderophore were detected, being present

Table 21. Total Bacteria Recovered and Detection of Bacterial
Siderophores from the Kidney of *Salmo gairdneri* Infected
with *V. anguillarum* strain 775

Inoculum of <u><i>V. anguillarum</i></u> strain 775 (c.f.u. per fish)	Viable bacteria Recovered from kidney	Siderophores Detected ^(a)
10^9 cells	3×10^3 (n = 2)	-
10^4 cells	4×10^4 (n = 2)	+
10^2 cells	5×10^5 (n = 2)	‡

(a) Siderophores were detected by analysis of ethyl acetate extracts of homogenised kidney supernate on paper chromatography (see figure 36).

(-) No bacterial siderophores were detected.

(+) Siderophores were detected in only one kidney sample, the other sample being negative.

(‡) Bacterial siderophores were detected in both kidney samples.

Figure 38. Paper chromatography of ethyl acetate extracts of tissue homogenates of Salmo gairdneri infected with V. anguillarum strain 775.

Paper chromatography of ethyl acetate extracts of tissue homogenates of Salmo gairdneri infected with 10^9 , 10^4 and 10^2 inocula of V. anguillarum strain 775 (a) viewed under ultraviolet illumination and (b) after staining with 1% (w/v) FeCl_3 and ammonia vapour.

Lanes 1 and 2, kidney homogenate, V. anguillarum strain 775, 10^4 inoculum.

Lane 3, kidney homogenate, V. anguillarum strain 775, 10^2 inoculum.

Lane 4, kidney homogenate, V. anguillarum strain 775, 10^9 inoculum.

Lane 5, kidney homogenate, uninfected control fish.

The common phenolate siderophore (I) and second phenolate siderophore (II) are marked in figure (b).

(a)



(b)

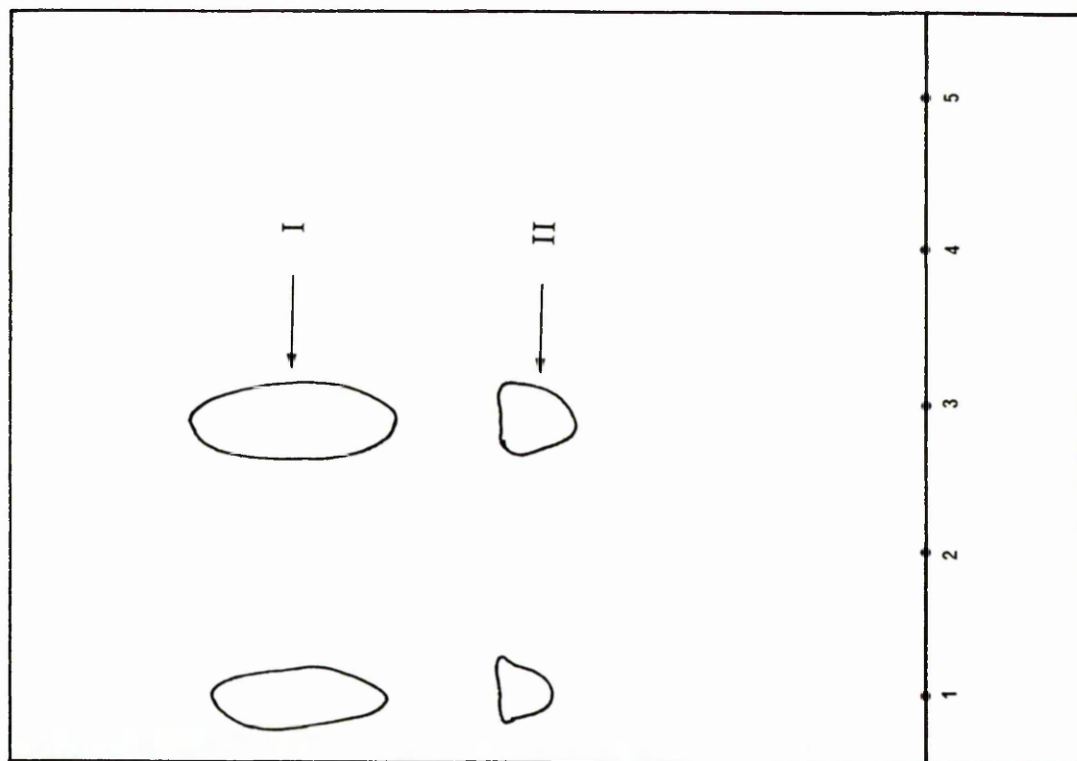


Table 22. The Effect of Freezing on the Total Number of Bacteria
Recovered from the Kidney of *Salmo gairdneri* Infected
with *V. anguillarum* strain 775

Kidney sample	Viable bacteria recovered from ^(a) the kidney
Removed from fish immediately after death	4.3 (\pm 0.2) $\times 10^5$
Removed from fish which had been frozen immediately on death	4.0 (\pm 0.1) $\times 10^5$

Values are mean \pm standard error of the mean for n=3; pure cultures of the *V. anguillarum* strain 775 were recovered from the kidneys of 3 fish in each experiment.

(a) Inoculum of 10^4 c.f.u. for each fish.

Table 23. Total Bacteria Recovered from the Kidney and Spleen of
Salmo gairdneri Infected with Vibrio anguillarum

<u>V. anguillarum</u> ^(a) strain	Viable Bacteria Recovered from	
	Kidney	Spleen
775	$5.0(\pm 0.3) \times 10^5$	5.0×10^4 (n = 1)
NCMB6	$5.1(\pm 0.1) \times 10^6$	5.0×10^5 (n = 2)
91079	$4.0(\pm 0.1) \times 10^6$	5.0×10^5 (n = 2)

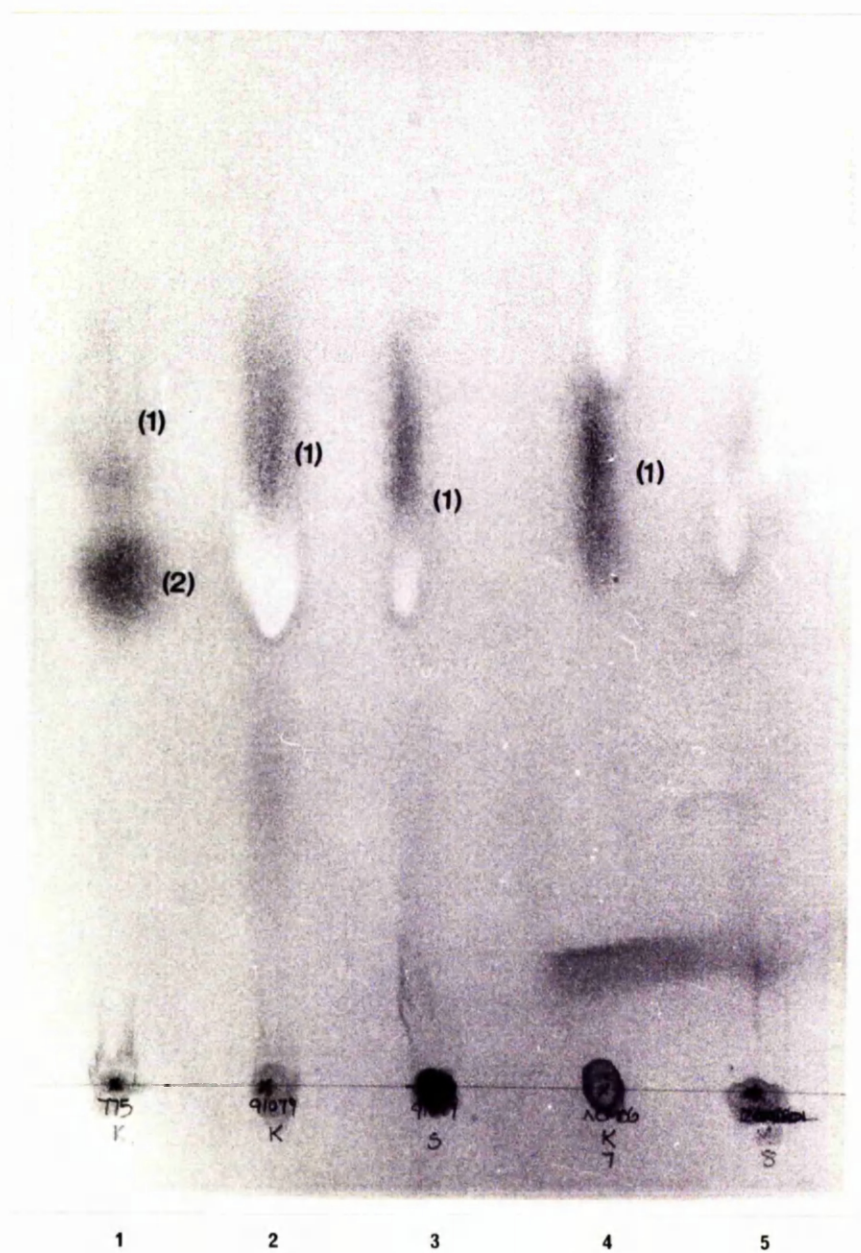
Values for the kidney are mean \pm standard error of the mean for n = 4; pure cultures of the input strain of V. anguillarum were recovered from the kidneys of 4 fish in each experiment. For the spleens, only 1 or 2 were recovered from each group as spleen liquefaction commonly occurred during infection.

(a) Inoculum of 10^2 c.f.u. for each fish.

Figure 39. Paper chromatography of ethyl acetate extracts of tissue homogenates of Salmo gairdneri infected with V. anguillarum.

Lane 1, kidney homogenate, V. anguillarum strain 775; lane 2, kidney homogenate, V. anguillarum strain 91079; lane 3, spleen homogenate, V. anguillarum strain 91079; lane 4, kidney homogenate, V. anguillarum strain NCMB6; lane 5, kidney homogenate, uninfected control fish.

The chromatogram was stained with 1% (w/v) FeCl_3 .
The common phenolate siderophore (1) and second phenolate siderophore (2) are marked.



in kidneys of all 4 fish and in the 2 available spleen samples. Comparison by paper chromatography of the siderophores produced in vivo and in vitro by the 3 V. anguillarum strains showed that the colour of fluorescence under UV light, colour of complex produced on reaction with iron and ammonia vapour and R_f values were indistinguishable. It was considered that the compounds produced in vivo were the same as those produced in vitro.

8. Relationship Between Serology, Plasmid Content and the Antigenic Components in the Cell Envelope of V. anguillarum

Serological analysis of the strains used in this study indicated a relationship between the group J-0-3 (Ezura et al., 1980) and the carriage of a plasmid (40-50mD) by strains V. anguillarum 775, 1197 and 4979 (table 24). Previous investigations have suggested that the major component in the cell envelope of V. anguillarum involved in the serology of this bacterium is LPS (Johnsen, 1977; Chart & Trust, 1984). Therefore, the relationship between the LPS isolated from the 10 V. anguillarum strains and the serological grouping of these strains was investigated by the electroblotting technique with crude antisera to both V. anguillarum strains 775 and NCMB6 (as described in section 3.3).

8.1 Comparison of LPS from V. anguillarum strains 775 and NCMB6

When the LPS of V. anguillarum strains 775 and NCMB6 were compared by SDS-PAGE, there were major differences in the patterns obtained (Figure 40). To determine whether this difference contributed to the serotyping of V. anguillarum crude antisera containing immunoglobulins to LPS of either strains 775 or NCMB6 were used in electro-

Table 24. Comparison of Plasmid Content and Serotype of Ten
V. anguillarum Strains

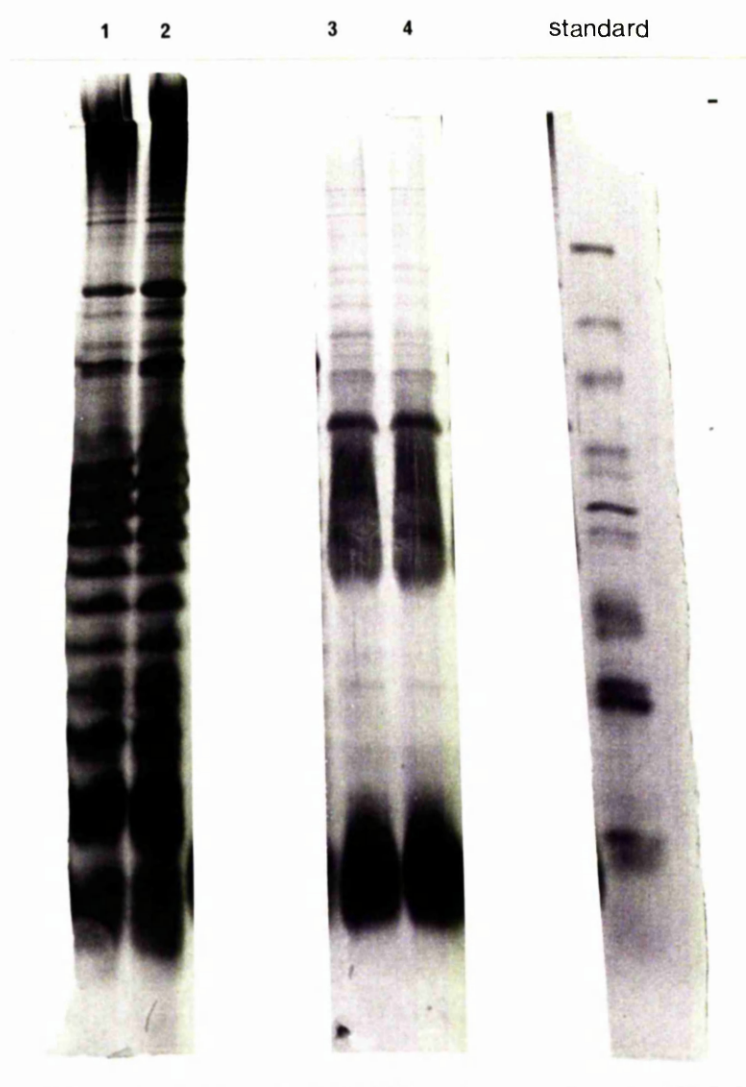
<u>V. anguillarum</u>	Serotype ^(a)	Plasmid Content
strain	(Ezura <u>et al.</u> , 1980)	(size in mD)
NCMB6	J-0-1	-
775	J-0-3	+ (47mD)
636	J-0-1	-
827	J-0-1	-
1197	J-0-3	+ (45-50mD)
1445	J-0-1	-
91079	J-0-1	-
2981	J-0-1	-
4979	J-0-3	+ (45-50mD)
5679	J-0-1	-

(a) Serotype groups determined by T.H. Birkbeck (unpublished results).

Figure 40. SDS-PAGE of LPS from V. anguillarum strains 775 and NCMB6.

LPS from V. anguillarum strains 775 and NCMB6 was separated by SDS-PAGE and bands were visualised with silver staining.

Lanes 1 and 2. LPS from V. anguillarum strain 775;
lanes 3 and 4, LPS from V. anguillarum strain NCMB6.



blotting experiments to determine antigenic similarities and differences in the LPSs of other V. anguillarum strains.

When the LPS of ten V. anguillarum strains were compared after electroblotting on nitrocellulose paper with crude antiserum to V. anguillarum 775, those from three strains (V. anguillarum 775, 1197 and 4979) reacted strongly with this antiserum (figure 41(a)). All three strains contain a plasmid (40-50mD) and were all of serotype J-0-3. With crude antiserum raised against strain NCMB6, all strains showed some degree of cross-reaction (figure 41(b)).

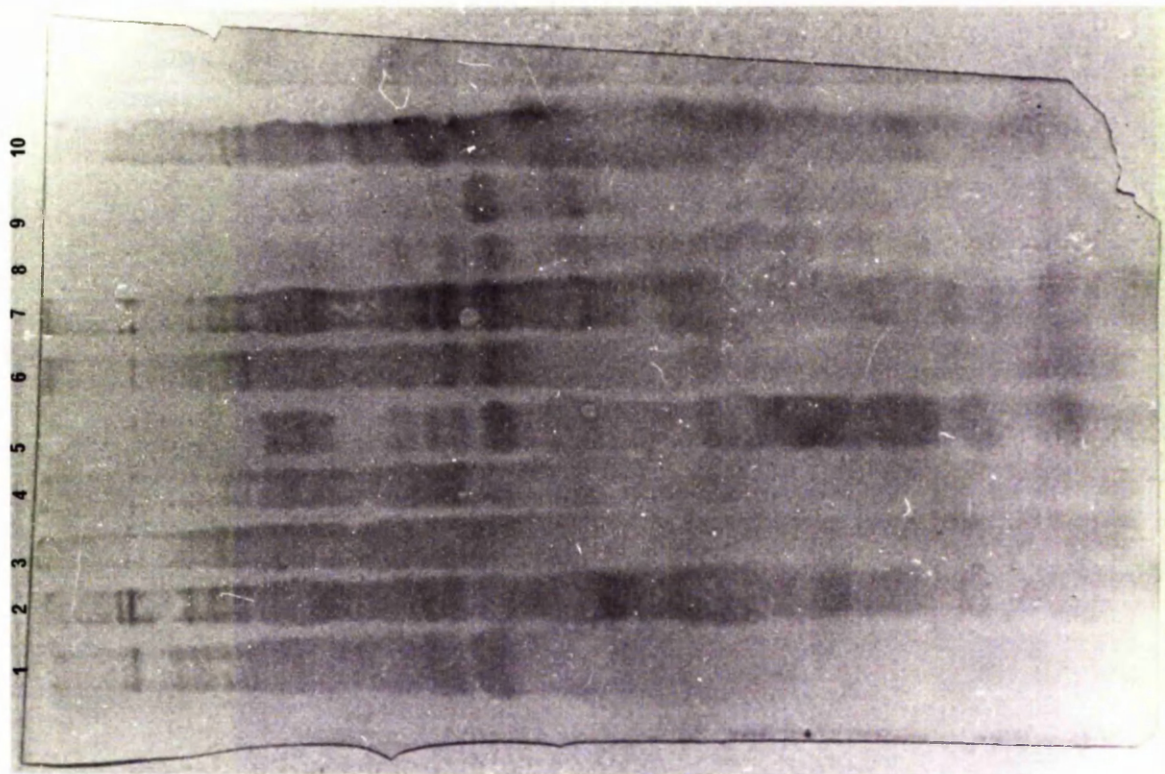
The crude antiserum to V. anguillarum 775 was then adsorbed with LPS until no precipitin reaction occurred in gel diffusion. This adsorbed antiserum did not react with LPS after electroblotting onto nitrocellulose paper, but reacted with at least two proteins in the cell envelope fractions isolated from all ten strains as well as a further component in strains 775, 4979 and 1197 which ran as a streak on the top part of the polyacrylamide gel (Figure 42). This envelope component may represent an additional antigen specific to serotype J-0-3.

Figure 41. Immunoblotting of LPS from V. anguillarum.

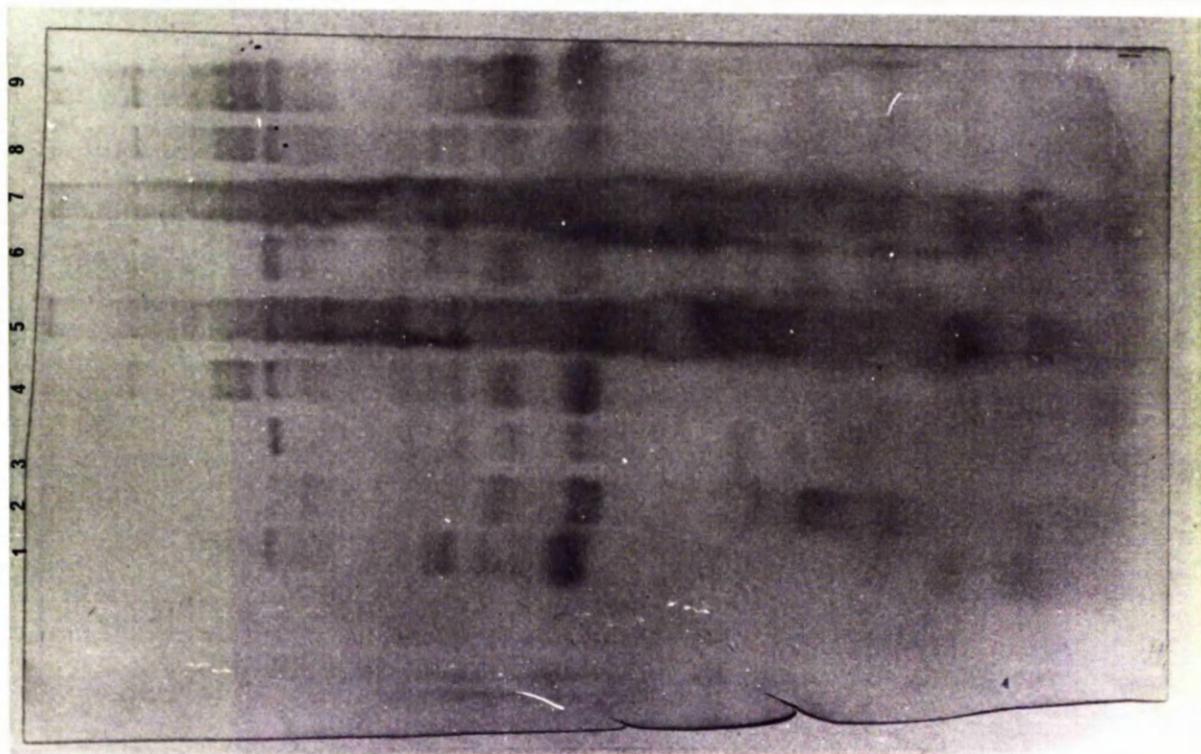
Nitrocellulose blot analysis of LPS from
V. anguillarum separated by SDS-PAGE and reacted with (a)
crude antiserum to V. anguillarum strain 775 and (b) crude
antiserum to V. anguillarum strain NCMB6.

figure (a). Lane 1, V. anguillarum strain 2981; lane 2,
V. anguillarum strain 91079; lane 3, V. anguillarum strain
827; lane 4, V. anguillarum strain 1445; lane 5,
V. anguillarum strain 4979; lane 6, V. anguillarum strain
5679; lane 7, V. anguillarum strain 1197; lane 8,
V. anguillarum strain 636; lane 9, V. anguillarum strain
NCMB6.

figure (b). Lanes 1-8, as above; lane 9, V. anguillarum strain 775;
lane 10, V. anguillarum strain NCMB6.



(b)



(a)

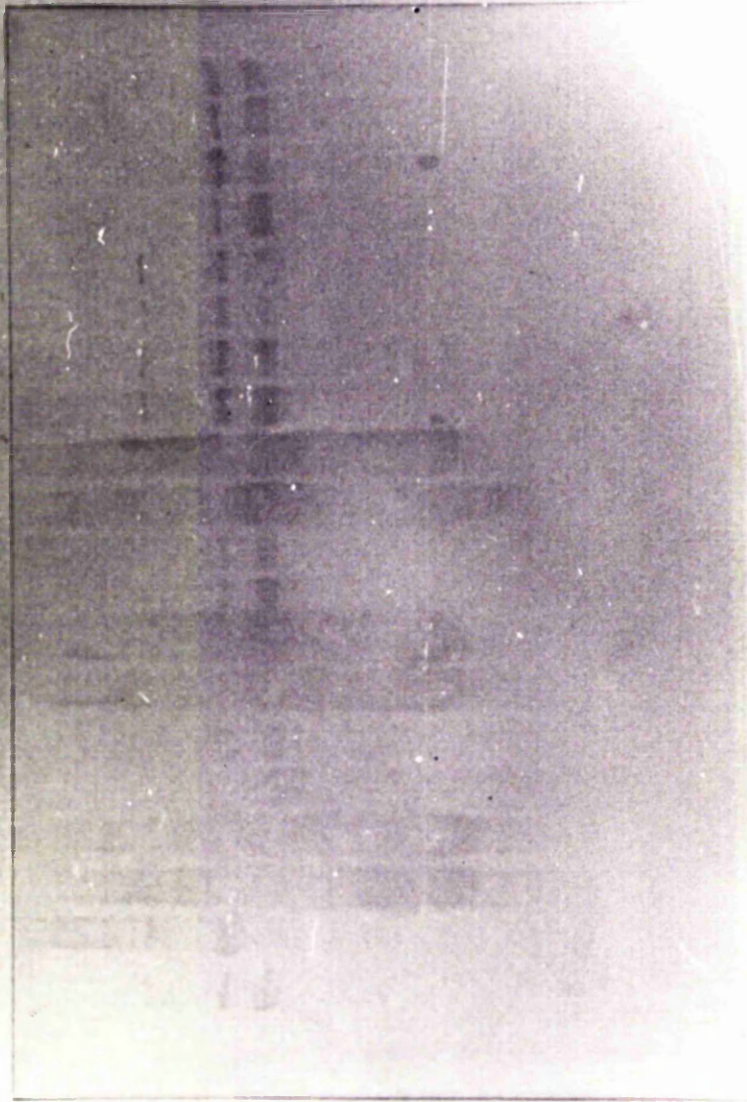
Figure 42. Immunoblot of 10 V. anguillarum strains with antiserum to a cell envelope fraction of V. anguillarum strain 775 absorbed with LPS of V. anguillarum strain 775.

The 20 cell envelope fractions from 10 V. anguillarum strains were obtained by growth of each strain in NBST medium (left lane of each pair of lanes) or in NBS (right lane of each pair of lanes).

Nitrocellulose blot analysis of cell envelope fractions from V. anguillarum separated by SDS-PAGE and reacted with absorbed antiserum to V. anguillarum strain 775 (absorbed with LPS from strain 775).

Lanes 1 and 2, V. anguillarum strain 5679; lanes 3 and 4, V. anguillarum strain 4979; lanes 5 and 6, V. anguillarum strain 2981; lanes 7 and 8, V. anguillarum strain 775; lanes 9 and 10, V. anguillarum strain 91079; lanes 11 and 12, V. anguillarum strain 1197; lanes 13 and 14, V. anguillarum strain 1445; lanes 15 and 16, V. anguillarum strain 827; lanes 17 and 18, V. anguillarum strain NCMB6; lanes 19 and 20, V. anguillarum strain 636.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



Discussion

1. Plasmid Associated Iron-Uptake Systems in *Vibrio anguillarum* and other *Vibrio* species

The importance of the aerobactin plasmid-mediated iron-uptake system during infection in invasive strains of *E. coli* (Williams, 1979) led to the discovery by Crosa (Crosa et al., 1977; Crosa, 1980) of an association between plasmid carriage and virulence in *V. anguillarum* and subsequently to the discovery that an iron-sequestering system was important in the pathogenesis of vibriosis in fish.

To determine whether a plasmid-associated iron-uptake system was widespread among pathogenic *Vibrio* species isolated from fish and oysters from different geographical locations, 23 strains of various *Vibrio* species were tested for the presence of plasmids, in particular, a plasmid class similar to pJMI. From the results no correlation was apparent between plasmid carriage and the ability to grow under iron-limiting conditions as only 3 *V. anguillarum* strains, 1197, 775 and 4979, contained a plasmid of similar molecular weight (45-50 Mdal), and 20 out of the 23 strains were able to grow in an iron-limiting medium (NBS medium plus transferrin). These preliminary results suggested that at least two iron-uptake systems were present in the 23 strains of *Vibrio* species, one plasmid-mediated (pJMI) and the other chromosomal-mediated.

Tomalsky et al. (1985) found a plasmid-mediated iron-uptake system in *V. anguillarum* isolated from infected turbot in Northern Spain indicating that such systems were not restricted to isolates from the North West Pacific. The strains in this study were geographically more diverse, number 1197 being isolated from a salmonid fish in Scandinavia and strain 4979 from moribund oysters in North Wales. The virulence of the latter strain in fish is unknown. Also, it is not

known how closely related are the plasmids of the strains 1197 and 4979 to plasmid pJMI.

The first molecular characterisation of marine Vibrios isolated from striped bass in Chesapeake Bay, U.S.A., showed that unlike the strains of V. anguillarum isolated in the Pacific Northwest, strains isolated from striped bass did not contain any plasmids. Nonetheless, these Atlantic Coast strains were similar to the Northwest isolates in that virulence was correlated with their ability to grow under conditions of iron limitation and, as in this present study, suggested the presence of a chromosomal-mediated iron-uptake system.

The small plasmid in V. anguillarum NCMB6 reported by Croza (1981) was not detected using any of the plasmid preparation techniques which were capable of detecting plasmids of low molecular weight and the plasmid may have been lost during prolonged subculture in the laboratory. It did not however diminish the virulence of strain NCMB6 (see Results, section 7).

2. Detection of Cell Envelope Proteins Associated with Iron Limitation

To investigate the iron-uptake systems of pathogenic V. anguillarum strains, ten serologically defined strains were chosen to determine the cell envelope proteins associated with iron limitation and to detect whether these proteins were antigenically similar in any or all of the strains.

Using the Improved EDTA extraction procedure up to three proteins associated with iron-limitation, depending on the strain of V. anguillarum, were detected in the cell envelope samples of cells grown in NBST. Of the reference strains, one protein of Mr = 69 kD was detected in the

cell envelope samples of V. anguillarum NCMB6 confirming the report of Crosa (1981) (see table 7) although the molecular weight was lower than previously reported. Two proteins were detected in the cell envelope of V. anguillarum 775 ($M_r = 73\text{kD}$ and 79kD). The 79kD protein probably corresponds to the OM1 protein encoded on virulence plasmid pJMI, and the 73kD protein to the OM3 protein which was of chromosomal origin (Crosa & Hodges, 1981; Actis *et al.*, 1985). In both cases however, the molecular weights were lower than expected from previous work. With seven other strains, V. anguillarum strains 636, 827, 91079, 2981, 4979, 5679 and 1197, either one or two cell envelope proteins were associated with iron-limitation and they were similar in molecular weight to the proteins found in the reference strains 775 and NCMB6. Only strain 1445 produced three proteins under iron-limiting conditions and these were of $M_r = 68, 72$ and 73k .

To determine whether these proteins were also immunologically related, absorbed antisera to V. anguillarum strains NCMB6 and 775 (see Materials and Methods, section 11(d)) were used to determine the cell envelope proteins associated with iron limitation using two dimensional crossed-immunoelectrophoresis and immunoelectroblotting onto nitro-cellulose paper.

Two dimensional immunoelectrophoresis of cell envelope fractions of V. anguillarum 775 and NCMB6, grown in either NBS or NBST and using absorbed antiserum A to the respective strains, detected an additional precipitin arc in both strains when grown in NBST. These arcs could therefore be associated with iron-limitation, but this cannot be stated with certainty. Although each precipitin arc relates to a particular protein in the cell envelope of V. anguillarum and a reference library

could be determined for comparison of different strains, the technique was not suitable for detection and differentiation of proteins associated with iron-limitation. The amount of antiserum required for each experiment also limited the usefulness of this technique.

The second technique examined, immunoelectroblotting, was more sensitive than C.I.E. Using absorbed antiserum B to V. anguillarum 775, four bands were visualised on the nitrocellulose paper with cell envelope fractions of strain 775 grown in NBST, but only two bands were seen from cell envelope fractions of cells grown in NBS. These two extra bands corresponded to the 73kD and 79kD molecular weight proteins and appeared to be induced only under conditions of iron-limitation. The two proteins common to both samples may be the major antigenic proteins in the cell envelope of V. anguillarum.

When cell envelope proteins of V. anguillarum strain NCMB6 grown in NBS and NBST were visualised on nitrocellulose paper using absorbed antiserum A to strain NCMB6, a wide range of protein bands were detected, in particular, a band corresponding to the 69kD molecular weight protein associated with iron-limitation. This protein band was present on nitrocellulose paper in both cell envelope preparations, but was enhanced when strain NCMB6 was grown under iron-limiting conditions. This suggests that the protein associated with iron-limitation in strain NCMB6 was a constitutive protein which was expressed at a higher level under conditions of iron-limitation. Since this 69kD protein was present in low concentrations in the cell envelope preparations of strain NCMB6 grown in NBS, further absorption of antiserum A to NCMB6 resulted in loss of antibodies to the 69kD protein. In comparing the two strains, V. anguillarum 775 and NCMB6, strain 775 appeared to induce two cell

envelope proteins under iron-limiting conditions whereas strain NCMB6 produced an increased amount of a constitutive protein.

To compare the cell envelope proteins associated with iron limitation in all ten strains of V. anguillarum, reactions with absorbed antiserum B to strain 775 were done on nitrocellulose paper as this antiserum appeared to be more specific for proteins associated with iron limitation. When cell envelope fractions, prepared from cells grown in NBST were compared, at least one protein produced by all ten strains was antigenically similar to the 73kD molecular weight protein in strain 775, although this protein did appear in smaller amounts in strains NCMB6, 636, 827, 1445, 2981, 4979⁹¹⁰⁷⁹ and 5679 grown in NBS (see figure 24). Therefore, the protein associated with iron limitation in the cell envelope of the above strains appeared to be a constitutive protein similar to the 69kD protein in NCMB6. Strains 5679, 1197 and 775 all produced two antigenically similar proteins of which the higher molecular weight protein was induced only under iron-limiting conditions. The lower molecular weight protein was also induced in strains 1197 and 775 only when the iron concentration was limited, but appeared to be a constitutive protein in 5679.

These results, in combination with those on p. 167, suggest that two independent iron-uptake systems exist in V. anguillarum, one system involving a constitutive protein receptor (69-73kD molecular weight) present in increased concentrations during iron limitation and the other system, involving an iron-limiting, inducible protein (78-79kD molecular weight). This induced protein, present in strains 775, 1197 and 5679, may be the OM2 protein usually encoded on plasmid pJMI, since two of the three strains carry a similar molecular weight plasmid. The OM2 protein has previously been shown

to be antigenically similar in V. anguillarum 775 (pJMI) and V. anguillarum strains isolated from diseased turbot in Spain which carried a plasmid of similar molecular weight to pJMI (Tolmasky et al., 1985).

The lower molecular weight protein(s) (constitutive or induced) could correspond to the chromosomal-mediated OM3 protein. This lower molecular weight is presumably associated with a chromosomal iron-uptake system although no antigenically similar chromosomal protein associated with iron limitation in V. anguillarum has been previously reported.

The two other techniques used to detect iron-binding proteins in V. anguillarum (detection of ⁵⁹Fe labelled proteins from whole cell preparations and detection of iron-binding proteins using Ferene S - see Results section) gave inconclusive results and were not continued in this present study.

3. Production of Three Different Siderophore Types by Strains of Vibrio anguillarum

The production of siderophores by several Vibrio species, V. anguillarum, V. cholerae, V. fluxialis and V. vulnificus has been well documented (Payne & Finkelstein, 1978; Simpson & Oliver, 1983; Andrus et al., 1983; Actis et al., 1986) and phenolate components have been detected with all these Vibrio species when cultured in iron-limiting or iron-depleted media. Of these phenolate compounds, anguibactin, a phenolate siderophore produced by V. anguillarum 775 (pJMI) and encoded on the plasmid pJMI has been extensively studied and characterised (Actis et al., 1986) although its structure has yet to be defined. A chromosomal-mediated siderophore, produced by V. anguillarum had not been shown prior to this work.

This present study showed that V. anguillarum produced three different iron-binding compounds and that the synthesis of these compounds was strain-dependent and could be influenced by the choice of iron-limited culture medium. The phenolate compound produced by all strains, "the common phenolate siderophore", reacted positively in the colorimetric Arnow assay but did not exhibit the classic blue fluorescence under ultra-violet illumination, or the dark blue complex on reaction with iron, of a phenolate siderophore or of dihydroxybenzoic acid, a precursor of enterochelin (Neilands, 1984). Such reactions are usually indicative of phenolate siderophore production, however this iron-binding compound (produced by all ten strains) exhibited a pink/lilac complex with iron and ammonium suggesting that this compound requires (NH_4^+) groups to complex with iron and is perhaps associated with the chromosomal iron-transport system of V. anguillarum.

The second phenolate compound, produced by V. anguillarum strains 775, 1197 and 4979, reacted with the characteristics of a phenolate siderophore. Since this siderophore was produced by strains which either contained plasmid pJMI or a plasmid of similar molecular weight it was assumed to be anguibactin. This iron-binding compound was not produced when the strains were cultured in TSM, however on addition of transferrin to the medium, synthesis of the second phenolate siderophore was detected, suggesting that either the production of this siderophore required the presence of an iron chelator or that the amount of free ferric iron present in TSM repressed the expression of the iron-transport system involving the second phenolate siderophore (see table 9).

The third iron-binding compound, the hydroxamate compound

produced by V. anguillarum strains NCMB6, 636 and 1445, was detected only on culture in TSM and consequently may only be a by-product of metabolism when succinate is the sole source of carbon. Hydroxamate siderophores are not commonly produced by Vibrio species and therefore its importance in the iron-transport system of V. anguillarum is unclear.

The Vibrio strains were therefore placed into one of three groups depending on the siderophore-types produced under conditions of iron limitation. Transport of ^{55}Fe into the cells only occurred with supernate of either the homologous strain or one of the same group, indicating that separate iron-transport systems exist for the three different siderophores.

Using competitive ^{55}Fe -Uptake assays the relative affinities for iron of the three siderophores were estimated as follows:

Second Phenolate	>	First Phenolate	>	Hydroxamate
siderophore		siderophore		siderophore

This relative affinity for iron may indicate a selective advantage to strains 775, 1197, 4979 and other strains able to produce the second phenolate siderophore when there is competition for available ferric iron.

Other microbial iron chelators, desferal and aerobactin inhibited the uptake of ^{55}Fe by each Vibrio strain with and without the homologous supernate. Whether this indicates that both desferal and aerobactin have a higher affinity for iron than any of the siderophore types produced by V. anguillarum or whether in the presence of one of these chelators V. anguillarum does not use a siderophore-dependent iron-uptake system, is unclear. However, when grown under iron-limiting conditions in the presence of desferal, no V. anguillarum siderophore-type

could be detected and the only iron-binding compound present in culture supernate was desferal. This suggests that V. anguillarum can obtain ferric iron from desferal by either direct uptake or by degradation of desferal to release iron. The inhibition by desferal of ^{55}Fe -Uptake by Vibrio strains indicates that the first hypothesis is invalid. Therefore a second mechanism to obtain ferric iron in iron-limiting conditions, independent of the siderophore-dependent iron-transport system, may be present in V. anguillarum and may involve the production of specific extracellular proteins or enzymes.

4. The Isolation of Siderophores from the Kidney and Spleen of Rainbow Trout (*Salmo gairdneri*) Infected with *Vibrio anguillarum*

Enterochelin, the siderophore produced by E. coli, has been isolated from the peritoneal washings of infected guinea pigs (Griffiths & Humphreys, 1980) and the importance of enterochelin and other iron-transporting compounds as virulence determinants of E. coli and Salmonella typhimurium in experimentally infected animals has also been shown (Rogers, 1973; Yancey et al., 1979). All these studies demonstrated that the ability to produce iron-transporting compounds is important in infections by enteric bacteria. However, the iron-vibriobactin transport system of V. cholerae is not required in infections of young mice and the organism must therefore obtain iron from a different source (Sigel et al., 1985).

Previous studies have shown that the loss of the virulence plasmid, pJMI of V. anguillarum, was correlated with attenuation in virulence of strain 775 (Crosa, 1980; Crosa et al., 1980) and indirect evidence for the production of siderophores in vivo by V. anguillarum has recently been obtained. Mixed infections of fish, in which

siderophore-deficient mutants of V. anguillarum 775 were cross-fed by the wild-type strain, strongly suggested that anguibactin was produced in vivo (Wolf & Crosa, 1986).

The demonstration in this study of the production of siderophores in vivo by three strains of V. anguillarum, is additional evidence that the presence of an iron-transport system, chromosomal-mediated or plasmid-mediated, is an important virulence determinant of V. anguillarum.

All strains tested (strains 775, NCMB6 and 91079) produced the common phenolate siderophore and in addition, strain 775 produced the second, apparently plasmid-associated phenolate siderophore (anguibactin). The in vitro uptake of ^{55}Fe showed that the anguibactin had a higher affinity for iron than the common phenolate siderophore and, as previously suggested, strain 775 may have a selective advantage during infections.

Although strain 775 produced two siderophore types in vivo, the attenuation in virulence caused by the loss of plasmid pJMI in this particular strain suggests that the common phenolate siderophore iron-transport system is defective in strain 775 since both strains NCMB6 and 91079 are virulent for fish yet possess only this siderophore-transport system. This confirms the importance of the plasmid pJMI in the virulence of V. anguillarum 775 and suggests that a chromosomal-mediated iron-uptake system exists in V. anguillarum and allows V. anguillarum strains not carrying plasmid pJMI to obtain ferric iron from the body fluids during infection.

This evidence contrasts the ways in which V. anguillarum and V. cholerae obtain iron in infections. Whereas V. anguillarum appears to produce either chromosomal- or plasmid-mediated siderophores in vivo and these siderophores apparently have a major role in the virulence of

this organism, V. cholerae obtains iron by a method independent of the iron-vibriobactin-transport system. It remains to be shown whether V. anguillarum can also obtain iron from a siderophore independent system in vivo, similar to the method in which the organism obtains ferric iron from desferal in an in vitro, iron-limited culture medium.

5. The Iron-Uptake Systems of *Vibrio anguillarum*

This present study has shown that under iron-limiting conditions, V. anguillarum strains can produce at least two iron-uptake systems; one plasmid-mediated (78-79kD membrane receptor and anguibactin) and the other chromosomal-mediated (69-73kD membrane receptor and phenolate siderophore). The ten strains tested should therefore be able to be placed into one of two groups, those producing the chromosomal-mediated iron-uptake system only and those producing both chromosomal- and plasmid-mediated iron-uptake systems. However, this is not the case; of the ten strains, V. anguillarum strains NCMB6, 636, 827, 1445, 91079 and 2981 produce only the chromosomal-mediated iron-uptake system and strains 775 and 1197 produce both systems, but strains 4979 and 5679 appear to express the complete chromosomal-mediated iron-uptake system and only partially, the plasmid-mediated iron-uptake system (see table 25).

V. anguillarum strain 4979, under iron-limitation, only produced the siderophore (anguibactin) but not the receptor (78-79kD) and carried a plasmid of 45-50mD molecular weight. This strain therefore appears to contain a defective plasmid, unable to express the membrane receptor protein and is a mutant of the plasmid-mediated iron-uptake system (sid⁽⁺⁾ rec⁽⁻⁾ plas⁽⁺⁾). V. anguillarum 5679, under iron limitation, produced only the membrane receptor of the plasmid-mediated iron-uptake system and did not contain a plasmid (mutant = sid⁽⁻⁾ rec⁽⁺⁾ plas⁽⁻⁾).

Table 25. Scheme Showing the Possible Relationship Between Strains
Carrying a Plasmid-Mediated Iron-Uptake System in *Vibrio*
anguillarum

<u>V. anguillarum</u>	Plasmid-Mediated Iron-Uptake System		
strain	Plasmid (a)	Receptor (b)	Siderophore (c)
775	+	+	+
1197	+	+	+
4979	+	-	+
5679	-	+	-

(a) Plasmids detected by the method of Kado and Lui (1981)

(b) Receptor of molecular weight 78-79kD.

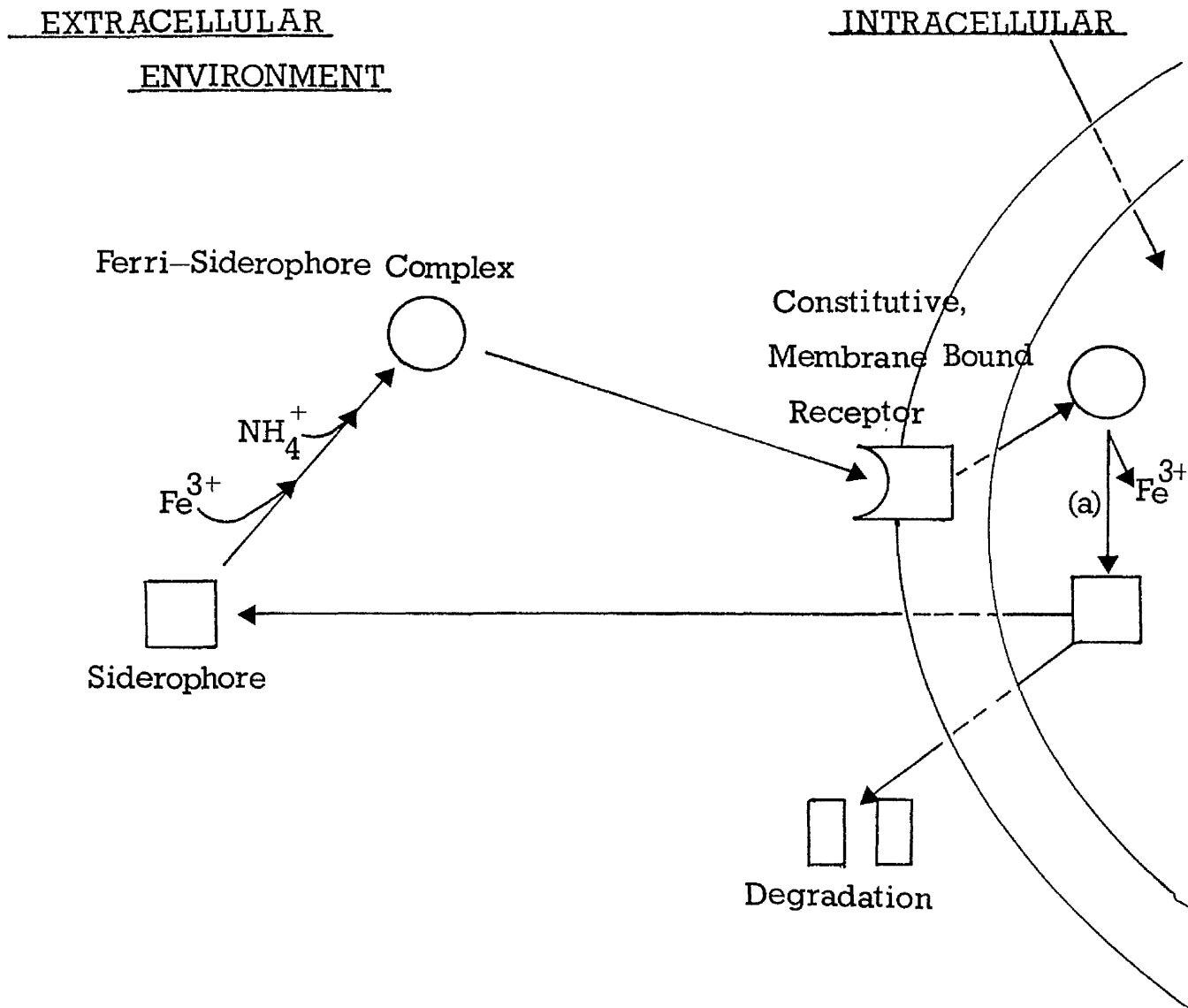
(c) Siderophore assumed to be anguibactin.

This strain could have a defective plasmid (similar to pJMI) integrated into the chromosome which can only express the membrane receptor. Previous investigations have shown that the aerobactin-transport genes (usually encoded on plasmid Col V) can commonly occur in the chromosome of different groups of human invasive strains of E. coli and other E. coli isolates (Valvano & Crosa, 1984; Bindereif & Neilands, 1985; Valvano et al., 1986) and the results of this present study suggest that a similar phenomenon can occur in some strains of V. anguillarum. The possibility that sequences of pJMI could be integrated into virulent plasmidless strains of V. anguillarum was also suggested by Crosa in 1980 (Crosa et al., 1980). The virulence of the two V. anguillarum strains, 4979 and 5679, in fish and the effect of a defective plasmid-mediated iron-uptake system on the virulence of the strains are still unknown.

The discovery by Crosa et al. (1980) that curing pJMI was correlated with an attenuation of virulence in V. anguillarum 775, suggests that the chromosomal iron-uptake system (shown in this study) is defective or ineffectual during infections of V. anguillarum 775, since both V. anguillarum strains NCMB6 and 91079 appear to be virulent in fish, but only produce the chromosomal iron-uptake-system. The requirement of two iron-uptake systems in strains 775 and 1197 suggests that the chromosomal iron-uptake-system in these strains are defective. This defect could occur in one of the stages of the chromosomal iron-uptake-system (a proposed schematic diagram of this iron-uptake system in V. anguillarum is shown in figure 43) as follows:

1. The siderophore may be unable to bind ferric iron under iron-limiting conditions.

Figure 43. A proposed schematic diagram of the
Chromosomal-Mediated Iron-Transport system
in Vibrio anguillarum



- (a) The exact mechanism of Reduction of the ferrisiderophore to release free Ferric iron intracellularly is unknown.

2. The ferri-siderophore complex may not be able to bind to the membrane bound receptor.

3. In V. anguillarum strains 775 and 1197, the receptor of the chromosomal-mediated iron-uptake system was inducible under iron-limiting conditions, unlike all other strains tested in which the receptor was constitutive and produced in higher concentrations during iron limitation. Therefore, the receptor in strains 775 and 1197 may be ineffectual and not produced in high enough concentrations.

4. The transport of ferric iron across the cell membrane into the cell from the outer membrane may be defective.

The defect in strain 775 is as yet unknown, however the results from this study suggest that the receptor may be defective in the chromosomal-mediated iron-uptake system.

From the fish infectivity experiments of this present study with V. anguillarum strains NCMB6 and 91079 it has been shown that the chromosomal-mediated iron-uptake system was expressed in vivo, suggesting that it was effective in obtaining iron during infections, although the phenolate siderophore produced in this iron-uptake system had the lowest affinity for iron among the three siderophores produced by V. anguillarum in vitro (see Results section 6). As the receptor for the chromosomal-mediated iron-uptake system is present in low concentrations during normal growth and production is increased during iron limitation, this may allow V. anguillarum to adapt quickly when conditions change and iron becomes limiting. With V. anguillarum 775, both phenolate-type siderophores were produced in vivo and the siderophore anguibactin produced by the plasmid-mediated iron-uptake-system, having a higher affinity for iron than the phenolate-type siderophore of the

chromosomal-mediated iron-uptake system, may give V. anguillarum strain 775 a selective advantage if there was competition between Vibrio strains during mixed infections. The production of two siderophores by V. anguillarum could be compared to the production of enterobactin and aerobactin iron-uptake systems by enteric bacteria (E. coli and Salmonella species) in vivo.

Although aerobactin has a lower affinity for iron than enterobactin (K_s values = 10^{23} and 10^{52} respectively; Neilands, 1981; 1983) in some cases the production of the aerobactin-iron-transport system may be more effective during infections. For example, the physiological and regulatory features of aerobactin result in a more effective provision of ferric ions for bacterial growth than does enterobactin and this may give a selective advantage to aerobactin-positive invasive strains of E. coli (Neilands, 1981; 1983; Williams & Carbonetti, 1986).

Previous work (Moore & Earhart, 1981; Griffiths et al., 1985; Chart & Griffiths, 1985) has also shown the presence of antibodies to the ferric-enterobactin receptor and enterobactin of most pathogenic E. coli strains in normal sera from rabbits, mice, guinea pigs and humans and this may question the importance of this iron-sequestering system in vivo. If antibodies to either the receptors or siderophores of the plasmid- or chromosomal-mediated iron-uptake systems in V. anguillarum exist in fish, then this may explain the importance of the two iron-sequestering systems in V. anguillarum.

6. Antigenic Components in the Cell Envelope of Vibrio anguillarum

Serological studies have demonstrated at least six serotypes of V. anguillarum (see table 1). The major surface antigens of V. anguillarum appear to be the lipopolysaccharide and several outer

membrane proteins (Johnsen, 1977; Chart & Trust, 1984). This present study suggests an additional component in the cell envelope of V. anguillarum may be important in the serology of the organism.

When the LPS of V. anguillarum strains 775 and NCMB6 were compared by SDS-PAGE two distinct morphologies were exhibited and this confirms previous reports by Chart & Trust (1984). These two strains also differed in serological groups, strain 775 belonging to group J-0-3 and strain NCMB6 to group J-0-1 (according to serogrouping of Ezura et al., 1980). The cell envelopes of the ten V. anguillarum strains were compared by immunoelectroblotting with rabbit antiserum raised against cell envelopes of V. anguillarum 775 and 3 strains (V. anguillarum 775, 1197 and 4979) gave a strong reaction with this antiserum. These strains were all of serotype J-0-3 and contained plasmids of similar molecular weight. With antiserum against strain NCMB6, all strains showed some degree of cross-reaction and when antiserum was absorbed with LPS it still reacted with a further component in strains 775, 1197 and 4979 and with at least two proteins in the cell envelope fractions of all ten strains.

The cross-reactive proteins have been previously reported by several workers (Schiewe & Hodgins, 1977; Buckley et al., 1981; Chart & Trust, 1984). One protein was a porin outer membrane protein which was transmembrane, peptidoglycan associated and surface exposed (Buckley et al., 1981). The other two proteins are major antigenic proteins (Mr = 49-51 kD) and are found in many V. anguillarum strains isolated from different geographical sources (Chart & Trust, 1984).

LPS is widely reported to be the major antigenic component in V. anguillarum and the most important component in any serological study.

However, some workers have suggested that other components may be involved (Johnsen, 1977; Schiewe & Hodgins, 1977; Sørensen & Larsen, 1986). This present study also indicates an additional component in the cell envelope of V. anguillarum which is apparently associated with the serogroups of V. anguillarum. The nature of this component is unknown but the streaking phenomenon seen on nitrocellulose paper after immunoelectroblotting is characteristic of carbohydrate or glycoproteins. There are several possible sources of carbohydrate in the cell envelope.

1. Glycoproteins are commonly present in the cell envelope of gram negative bacteria (Owen et al., 1982) although none have been previously reported in the cell envelope of V. anguillarum.

2. Polysaccharides are also found in peptidoglycan. However in gram negative bacteria peptidoglycan is not associated with the surface of the cell and would not therefore be likely to be involved in immunological reactions at the cell surface.

3. Polysaccharide capsules have been recently reported in V. anguillarum by Sørensen & Larsen (1986). Since a capsule usually surrounds the cell surface and it would be an obvious candidate for a major immunological component in serological studies. The Lancefield Groupings in the serology of streptococci is the primary example of the importance of different carbohydrates present in capsular material when studying the serology of an organism.

7.

Future Studies

The plasmid pJMI of V. anguillarum strain 775 has been extensively studied by Crcsa and his colleagues (Crosa et al., 1980; Walter et al., 1983) and it seems from the present study that similar plasmids may be carried by two further strains of apparently quite different origins. More detailed comparison of these plasmids to determine the degree of similarity between them could be done by examining restriction endonuclease patterns using for instance ECoRI, BamHI and HindIII. Such patterns could be compared to those of plasmid pJMI and related plasmids discovered by Tolmasky et al. (1985) in Spain and would be of interest in assessing how widespread is the distribution of such plasmids.

All three strains which carried plasmids were of serotype J-0-3 but it is not known whether there is any direct relationship between between the two properties. Attempted transfer of plasmid pJMI to other vibrios would be of interest in a number of ways. Firstly, to test whether carriage is restricted to vibrios of particular species or serotype. Secondly, to determine whether carriage of the plasmids affects the virulence of these vibrios.

The ten strains of V. anguillarum used in this study have been placed into one of three groups according to the production of siderophores in different culture media. It would be of interest, therefore, to calculate the LD₅₀ dose of each strain in rainbow trout and determine any relationship between LD₅₀ values, siderophore production and the efficiency to take up radioactive ⁵⁵Fe.

From the preliminary in vivo experiments in this study, it has been shown that siderophores produced by V. anguillarum were produced in vivo during infection. In future work, cross-feeding experiments,

similar to those done by Wolf and Crosa (1986) could be carried out using V. anguillarum strain 775 and the "mutant" strain 5679 which lacks the siderophore, anguibactin. The virulence of strain 5679 in fish is still unknown, but it would be of interest to show whether mixed infections with strains 775 and 5679 have any effect on the multiplication and virulence of the "mutant" strain in vivo and determine whether the presence of strain 775 enhances the recovery of strain 5679 from infected tissue.

To determine whether V. anguillarum strain 5679 carries a sequence of pJMI integrated into the chromosome, copy DNA (cDNA) of plasmid pJMI would be prepared to test for the presence of pJMI in the chromosome by ³²P-hybridisation experiments. Tolmasky et al. (1985) have suggested that pJMI could be spread by recombinational processes, involving transposition elements, after finding iron-uptake genes of pJMI in turbot strains of V. anguillarum carrying pJMI-like plasmids.

The nature of the apparent defect in strain 775 in transport of iron via the common phenolate siderophore merits further investigation. Curing of the plasmid pJMI, would remove the capacity for synthesis of both anguibactin and the outer membrane receptor proteins. Uptake ratios for ⁵⁵Fe in the presence of transferrin or siderophores would indicate whether a high-affinity iron-transport system via the common phenolate siderophore is still operative.

Chemical characterisation of the common phenolate siderophore is essential for further comparison of the iron-transport mechanisms of different vibrios.

With oyster-virulent strains of Vibrio, the importance of an iron-uptake system is unknown. However, this study showed that of

23 Vibrio species, only three strains, V. anguillarum 1337 and Vibrio sp 1338 and B51 could not grow in the presence of transferrin. These strains were originally isolated from diseased oysters and indicates that the ability to grow under iron limitation is not necessarily a virulence determinant in oysters. The pathogenicity of vibriosis is not yet understood, but a detailed study in the importance of iron in the virulence of oyster-pathogenic Vibrio would be of great significance in a future study.

Finally, despite the development of vaccines which appear to be effective in fish, vibriosis is still of great economic importance in marine aquaculture. The nature of the other virulence factors of V. anguillarum is unclear and a systematic study of virulence determinants of the organism, for example by transposon mutagenesis, would be desirable to better understand the mechanisms of pathogenesis in vibriosis.

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Appendices

Appendix 1MediaA. Nutrient Broth and Salt (NBS Medium)

Oxoid Nutrient Broth No. 2	25g
NaCl	15g
Distilled Water	1000ml

The medium was autoclaved at 121°C for 15 minutes.

B. NBS Medium plus Transferrin (NBST)

Human transferrin (Sigma) was added to NBS to a final concentration of 500 μgml^{-1} and the medium was filter sterilised (0.45 μm Millipore filter membrane). The medium was kept at 25°C for 24h before use.

C. NBST Medium plus FeCl_3

To NBST, 105.42 mg l^{-1} FeCl_3 was added before resterilisation by filtration through a 0.45 μm Millipore filter. The medium was kept at 25°C for 24h before use.

D. NBS Medium plus Desferal

To NBS, desferal (Ciba-Geigy) was added to a final concentration of 500 μgml^{-1} and sterilised by filtration through a 0.45 μm Millipore filter. The medium was kept at 25°C for 24h before use.

E. Marine Vibrio Minimal Medium (VMM)

The medium was modified from Clowes and Hayes, 1968.

NH_4Cl	5g
NH_4NO_3	1g
Na_2SO_4	2g
K_2HPO_4	3g
KH_2PO_4	1g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1g
NaCl	15g
Distilled deionised water to	980ml

The medium was autoclaved at 121°C for 15 minutes and the following filter sterilised solution was added:

glucose	4g
thiamine	$2 \mu\text{gml}^{-1}$
aspartic acid	$20 \mu\text{gml}^{-1}$
histidine	$20 \mu\text{gml}^{-1}$
sodium citrate	0.294g
Distilled deionised water	20ml

F. VMM plus Transferrin (VMMT)

To VMM, human transferrin (Sigma) was added to a final concentration of $80 \mu\text{gml}^{-1}$ and the solution was sterilised by filtration ($0.45 \mu\text{m}$ membrane). The medium was kept at 25°C for 24h before use.

G. Tris Succinate Medium (TSM)

The medium was modified from Braun (1981).

NaCl	10g
KCl	3.7g
NH ₄ Cl	1.1g
CaCl ₂ .2H ₂ O	0.15g
Na ₂ SO ₄	0.142g
KH ₂ PO ₄	0.272g
MgCl ₂ 6H ₂ O	0.10g
Tris	12.1g
Sodium Succinate	10g
Distilled deionised water	800ml

The pH was adjusted to 6.8 with 1N NaOH. The medium was made up to 1 litre with distilled, deionised water and sterilised by filtration (0.45µm membrane).

H. TSM plus Transferrin (TSMT)

To TSM, human transferrin was added to a final concentration of 100 µg/ml and sterilised by filtration (0.45µm membrane). The medium was kept at 25°C for 24h before use.

J. Nutrient Agar and Salt (NAS)

Oxoid Nutrient Agar	28g
NaCl	10g
Distilled water	1000ml

The medium was autoclaved at 121°C for 15 minutes.

K. Thiosulphate/Citrate/Bile Salts Agar (TCBS)

TCBS Agar (Difco) 89g

Distilled water 1000ml

The medium was dissolved by heating at 100°C and dispensed into petri dishes and allowed to solidify.

Appendix IIAgarose Gel Electrophoresis and Plasmid PreparationsA. Stock Solutions(i) Lysis Buffer (Kado and Liu, 1981)

Tris	0.303g
Sodium dodecyl sulphate (SDS)	1.5g
Distilled water	70ml

The pH was adjusted to 12.4 with 1N NaOH and the final volume made up to 100ml with distilled water.

(ii) Sucrose, Triton-X-100, EDTA, Tris (STET) Buffer (Holmes and Quigley, 1981)

Sucrose	8g
Triton-X-100	5.3g
50mM Ethylenediaminetetraacetic acid (EDTA)	10 μ l
5mM Tris/HCl pH 8	5 μ l
Distilled deionised water to	100ml

(iii) Tris, EDTA (TE) Buffer pH 8.0 (Holmes and Quigley, 1981)

0.01M Tris	0.121g
1mM EDTA	0.037g
Distilled deionised water	70ml

The solution was adjusted to pH 8 with concentrated Hydrochloric acid and the final volume made up to 100ml with distilled, deionised water.

B. Electrophoresis Buffers(i) Tris Acetate Buffer pH 7.9 (Kado and Liu, 1981)

Tris	4.846g
EDTA	0.744g

The above was dissolved in distilled water, the pH adjusted to 7.9 with concentrated acetic acid and the final volume made up to 1 litre with distilled water.

(ii) Tris Borate Buffer pH 8.8 (Holmes and Quigley, 1981)

To make 1 litre of 10X Concentrated pH 8.8 with 1mM EDTA

Tris	108g
Boric acid	23.5g
EDTA	3.7g
Distilled water to	1000ml

C. Tracking Dye

Bromophenol Blue	7mg
SDS	0.7g
glycerol	3.3g

Tracking dye A (Kado & Liu, 1981). Tris acetate buffer was added to a final volume of 10ml.

Tracking dye B (Holmes & Quigley, 1981). Tris borate buffer was added to a final volume of 10ml.

Appendix IIIPolyacrylamide Gel Electrophoresis (Laemmli, 1970)A. Stock Solutions(i) Acrylamide/BIS

Acrylamide	30g
N,N-bis-methylene acrylamide	0.8g
Distilled water to	100ml

(ii) Lower Buffer (4X concentration) pH 8.9

Tris	18.1g
SDS	0.4g
Distilled water	70ml

The pH was adjusted to 8.9 with concentrated HCl and the final volume made up to 100ml with distilled water.

(iii) Upper Buffer (4X concentration) pH 6.8

Tris	6.06g
SDS	0.4g
Distilled water	70ml

The pH was adjusted to 6.8 with concentrated HCl and the final volume made up to 100ml with distilled water.

(iv) Temed (undiluted stock)(v) Ammonium Persulphate Solution

A 10% solution was made up freshly (50mg in 0.5ml distilled water).

(vi) Solubilising Buffer for Proteins

Glycerol	10ml
2-mercaptoethanol	5ml
SDS	3g
Bromophenol blue	0.01g
Upper buffer (1 in 8 dilution of (iii))	to 100ml

(vii) Solubilising Buffer for LPS (Tsai and Frasch, 1982)

Glycerol	10ml
Sucrose	20g
SDS	2g
2-mercaptoethanol	5ml
Bromophenol blue	0.01g
Upper Buffer (1 in 8 dilution of (iii))	to 100ml

(viii) Staining Solution

Coomassie Blue R250	1.25g
50% (v/v) methanol	454ml
Glacial acetic acid	46ml

(ix) Destaining Solution

Methanol	50ml
Glacial Acetic Acid	75ml
Distilled water	875ml

(x) Running Buffer pH 8.3

Tris	3.03g
Glycine	14.4g
SDS	1.0g
Distilled water to	1000ml

The pH was adjusted to 8.3 with concentrated HCl.

B. Slab-Gel Preparations(i) Lower Gel-Separating Gel

	12.5%	10%
Lower Buffer (4X)	10ml	10ml
Distilled water	13.4ml	16.6ml
Acrylamide/BIS	16.6ml	13.4ml

After degassing for 20 minutes the following were added:

Ammonium persulphate solution	200 μ l	200 μ l
Temed	20 μ l	20 μ l

(ii) Upper Gel-Stacking Gel (4.5%)

Upper Gel Buffer (4X)	2.5ml
Distilled water	6ml
Acrylamide/BIS	1.5ml

After degassing for 10 minutes the following were added:

Ammonium persulphate solution	30 μ l
Temed	20 μ l

C. Miscellaneous Stains(i) Ferene S Staining Solution

Ferene S	0.371g
Thioglycollic acid	1.712g
50% (v/v) methanol	902ml
Glacial acetic acid	92ml

(ii) Silver Staining Reagent

Ammonia solution	2ml
0.1N NaOH	28ml
20% (w/v) silver nitrate	5ml
Distilled water	115ml

D. Buffers for Protein and LPS Immunoblotting(i) Transfer Buffer

Tris	3.03g
glycine	14.4g
20% (v/v) methanol to	1000ml

(ii) Wash Buffer

Tris	2.42g
NaCl	29.2g
Distilled water to	1000ml

The pH was adjusted to 7.5 with concentrated HCl and Tween 20 (Sigma) was added to a final concentration of 0.1% (v/v).

(iii) Substrate

Tris	0.242g
NaCl	2.92g
Distilled water to	100ml

The pH was adjusted to 7.5 with concentrated HCl and 60 μ l of hydrogen peroxide (H_2O_2) was added. 4-chloro-naphthol (60mg, Biorad) was dissolved in 20ml methanol and added to 100ml of the above buffer immediately before use.

Appendix IVBuffers and Diluents(i) Barbitol-HCl Buffer for Electrophoresis pH 8.6

Sodium barbitone	4.12g
Distilled water to	1000ml

The pH was adjusted to 8.6 with concentrated HCl before adjustment to the final volume.

(ii) Buffer for Chemical Lysis and X-Press Method (Yamato et al., 1975; Owen et al., 1982).

1M Tris/HCl pH 7.8	10 μ l
NH ₄ Cl	0.32g
Magnesium acetate	0.214g
2-mercaptoethanol	50 μ l
Distilled water	70ml

After dissolution the final volume was adjusted to 100ml.

(iii) EDTA Extraction Procedure

Sodium ortho-phosphate (Na ₃ PO ₄ ·12H ₂ O)	9.5g
NaCl	4.383g
EDTA	1.681g

Dissolve the above in distilled water and adjust the pH to 7.4 with 1N NaOH and adjust the final volume to 500ml.

(iv) 0.1M Sodium Phosphate Buffer pH 8.0

Stock solutions:

A. 0.2M solution of monobasic sodium phosphate (27.8g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$
in 1 litre)

B. 0.2M solution of dibasic sodium phosphate (71.7g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$
in 1 litre)

The buffer was prepared by mixing 15.3ml of A and 84.7ml of B and
dilution to 200ml.

(v) Sodium Acetate Buffer pH 5.0

Stock solutions:

A. 0.2M solution of acetic acid (11.55ml in 1 litre)

B. 0.2M solution of sodium acetate (16.4g in 1 litre)

The buffer was prepared by mixing 14.8ml of A and 35.2ml of B and
dilution to 100ml.